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Grant Number: DAMD17-94-J-4260

TITLE: Identification and Genetic Mapping of Genes for Hereditary Breast Cancer and Ovarian Cancer in Families Unlinked to BRCA1

PRINCIPAL INVESTIGATOR: Susan L. Neuhausen, Ph.D.

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, Utah 84102

REPORT DATE: September 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 15, 1999	Final (22 Aug 94 - 21 Aug 99)

4. TITLE AND SUBTITLE
Identification and Genetic Mapping of Genes for Hereditary Breast Cancer and Ovarian Cancer in Families Unlinked to BRCA1

5. FUNDING NUMBERS
DAMD17-94-J-4260

6. AUTHOR(S)
Susan L. Neuhausen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Utah
Salt Lake City, Utah 84102

e-mail: susan@episun5.med.utah.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

A family history of breast cancer is a major risk factor for developing breast cancer, with estimates that up to 10% of breast cancer is due to a genetic predisposition. The original objective of this grant was to localize *BRCA2*. At the time the grant was funded, we had localized *BRCA2*, so we modified the aims to isolate *BRCA2*. Our collaborator on this grant, Dr. Stratton, isolated *BRCA2* at the end of 1995. The aims were modified to characterize *BRCA2*, including identifying mutations in high-risk breast cancer families, identifying *BRCA2* mutation carriers, investigating mutation origin of recurrent mutations, examining age-specific penetrance and risks of other cancers, and exploring other factors which may modulate risks of developing cancer in *BRCA2* mutation carriers. These aims were accomplished, resulting in 20 publications of research results and four reviews. Highlights include: cloning *BRCA2*; identifying founder mutations 6174delT in Ashkenazi Jews and 999del5 in Icelanders; investigating origins of recurrent mutations; identifying mutations and mutation carriers for studies of penetrance and risks of other cancers; examining the effect of oral contraceptive use on reducing risk of ovarian cancer; and the effect of bilateral prophylactic oophorectomy on reducing risk of breast cancer.

14. SUBJECT TERMS
Genetics, Hereditary, Genomic Search, Candidate Gene, Linkage Analysis, Gene Mapping, Humans, Anatomical Samples, Ovarian, Breast, Breast Cancer

15. NUMBER OF PAGES
182

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Susan L. Neuhausen 9-15-99

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION:

Ten percent of breast cancer may be due to a genetic predisposition. The initial goal of this grant was to clone *BRCA2* and that has been realized. We then moved forward to characterize this gene and its importance in familial breast cancer. Characterization involves identification of mutations within families, analysis of those mutations in the families to identify all mutation carriers, examination of age-specific penetrance for breast cancer, as well as risks of other cancers, investigation of recurrent mutations, and exploration of other risk factors which may modulate the risk of cancer in mutation carriers. Examination of founder mutations within populations such as the 999del5 in Icelanders and the 6174delT in Ashkenazi Jews may allow for better estimation of risk in these populations and directed genetic testing and counseling. Estimating the risks for developing breast and other cancers is important for counseling individuals who carry these high-risk mutations. By understanding which other risk factors modulate risk in these mutation carriers, we may be able to provide individual risk assessment, as well as provide clues for designing better therapeutics and preventative measures.

BODY:

The aims of this grant changed and were expanded as the project progressed. This was because the original goals were to localize and then clone *BRCA2*. We had localized *BRCA2* at the time this grant was funded and our collaborator on the grant, Dr. Stratton, cloned *BRCA2* during the second year of funding. We then expanded the aims to include characterization of *BRCA2*, which is described below. Therefore, I am describing the accomplishment of the aims as they changed over time.

The technical objectives from 8-94 to 2-96 were the following.

1. Construction of fine-scale genetic and physical maps in the *BRCA2* region. As part of our effort to isolate the *BRCA2* gene, detailed genetic, physical, and transcription maps were generated (Couch et al., 1996). A total of 14 polymorphic, short tandem repeats (STRs) were generated, many of which were utilized in the haplotyping studies described later.

2. Continue to ascertain families. We did not ascertain any additional large families as we had narrowed the region sufficiently that more families were not needed. However, we did continue to extend existing families to identify additional mutation carriers. This is described in more detail later.

3. Look for recombinants in *BRCA2*-linked families. We were able to identify key recombinants within our families to more closely localize the *BRCA2* gene. Key recombinants are described in Couch et al. (1996).

4. Study large *BRCA2*-linked families in order to estimate age- and site-specific cancer risks. We published a paper in 1995 (Goldgar et al., 1995) describing a very large *BRCA2*-linked kindred and a second manuscript examining cancer risks in two *BRCA2*-linked families in 1997 (Easton et al., 1997).

5. We constructed haplotypes using the 14 polymorphic markers we identified during this project. They allowed us to determine that the Icelandic families all shared the same haplotype and thus would have the same mutation. After isolation of the gene, the common founder mutation was identified (Thorlacius et al., 1996).

The objectives listed above were to develop the physical and genetic resources in order to clone the *BRCA2* gene. We accomplished these objectives, as our collaborator on this grant, Dr. Stratton, isolated the *BRCA2* gene (Wooster et al., 1995). Utilizing our genetic data and the physical map, we were able to describe the entire *BRCA2* gene (Tavtigian et al., 1996). *BRCA2* is a large protein of 3,418 amino acids and is dissimilar to *BRCA1* (Tavtigian et al.,1996).

With the cloning of the gene, we expanded our objectives (revised SOW of 2-6-96). The new aims were to 1) screen for *BRCA2* mutations in our collection of kindreds which are not due to *BRCA1*; 2) analyze *BRCA2* mutations in high-risk probands and their relatives in order to obtain age- and site-specific cancer risks, and expand families to identify all carriers; 3) analyze epidemiological and genetic risk factors in *BRCA2* mutation carriers; and 4) Perform haplotype analysis to study mutation origin.

Aim 1 (revised Statement Of Work of 2-6-96).
In the primary screening for mutations in the *BRCA2* gene, we concentrated on the families with a high probability of breast cancer due to *BRCA2*. Of 12 Utah families and an additional 6 families of collaborators, we identified 8 predisposing breast cancer mutations and 9 polymorphisms (S. Tavtigian et al., 1996). As part of expanding our set of *BRCA2* female mutation carriers, we continued to look for mutations in *BRCA2* in Utah high risk breast cancer families for which no *BRCA1* mutations had been identified. We examined the youngest individual with breast cancer from each of 40 Utah families by single strand conformational analysis (SSCA). In order to screen all the coding regions and intron/exon boundaries with overlapping amplicons, 75 primer pairs were made. We have now identified 17 Utah families with deleterious mutations and 5 families with missense mutations of unknown significance. Five families with clear linkage to *BRCA2* still have no identified mutation. No mutations were detected in an additional 25 families.

Mutations which result in a non-functional gene product can involve deletions, insertions, or rearrangements of portions of the DNA in the gene. For the five families linked to *BRCA2* for which no mutations had been identified by traditional sequencing of transcribed sequences and intron/exon boundaries, we looked for evidence of large deletions which would have been missed by sequencing. In addition to the 5 initial families, we included samples from 5 Utah families and from 4 families from our previous collaborator on this grant, Dr. Stratton at ICRF. Unfortunately, Dr. Stratton was only able to send 10 ug of DNA per sample, so that his samples were only examined with one restriction enzyme. Using published *BRCA2* sequences in Genbank, we constructed a restriction map of the entire *BRCA2* gene. Using 5 restriction enzymes and 14 probes, we probed Southern blots of DNA from early-onset breast cancer cases from each of these 5 families and looked for evidence of large (> 1 kb) deletions and/or rearrangements. If there was a large (> 1 kb) deletion or rearrangement in an individual, she

would have a different size band (variant) than the others on the Southern blot. We had many technical difficulties trying to make the probes with a sufficiently high radioactivity count in order to detect the signal (see the bands) on the Southern blots. We identified what appeared to be aberrant bands for several individuals with different restriction enzymes using different probes. However, we did not identify any consistent variants that were present on either two different restriction enzymes using the same probe (i.e., in the exact same region) or with two different probes in overlapping, adjacent regions, as would be expected if the variants were due to a deletion or rearrangement. The result was that we did not detect any mutations with this technique.

Aim 2 (revised Statement Of Work of 2-6-96).

A. Estimations of age and site-specific penetrances.
From the results of the previous aims, we had published a manuscript estimating age-specific penetrance based on data from two large *BRCA2*-linked families (Easton et al., 1997).The analysis of penetrance was performed using standard life-table estimates of gene carriers. As a member of the Breast Cancer Linkage Consortium (BCLC), we pooled our data with other collaborating scientists in order to obtain more accurate estimates of age-specific penetrance and of the contributions of *BRCA2* and *BRCA1* to high-risk breast cancer families (Ford et al., 1998). The contribution of *BRCA1* and *BRCA2* to inherited breast cancer was assessed by linkage and mutation analysis in 237 families with a minimum of four cases of breast cancer. Overall, cancer was linked to *BRCA2* in 32% of the families, *BRCA1* in 52% of the families, and no evidence in 16% of the families, suggesting that there are other genes predisposing to breast cancer. The penetrance of *BRCA2* (proportion developing breast cancer) was 28% by age 50 years and 84% by age 70 years. The lifetime risk is thus similar to what had been reported for *BRCA1*, although there is some suggestion of a lower risk in *BRCA2* mutation carriers <50 years of age.

In a second collaborative BCLC study, we examined site-specific risks in *BRCA2* mutation carriers from 173 families (BCLC, 1999). We found that there are statistically increased risks of developing prostate cancer at any age [relative risk (RR) = 4.65], prostate cancer at < 65 years (RR = 7.33); melanoma (RR = 2.58), gall bladder and bile duct cancer (RR = 4.97), pancreatic cancer (RR = 3.51), and stomach cancer (RR=2.59)(BCLC, 1999). Thus, it is important when counseling individuals in *BRCA2* families to discuss that they are also at increased risks of several other cancers in addition to breast and ovarian cancers.

B. Identify and collect additional *BRCA2* mutation carriers.
We have continued to sample within our *BRCA2* families and have identified 79 female mutation carriers with deleterious *BRCA2* mutations. The number of female mutation carriers by family with cancer status is shown in Table 1. We have also focused on obtaining up-to-date questionnaires from female *BRCA2* mutation carriers and have collected and entered 51 into the database. Questionnaires include medical history,

family history of cancer, reproductive history, oral contraceptive and hormone replacement use, and lifestyle factors including alcohol and tobacco use.

Table 1. Number of females with DNA samples carrying known deleterious *BRCA2* mutations

Kindred	Mutation	Total #	# BC	# OvC	# BC+OvC
2324	893delCC	4	1	1	1
1018	982del4	6	4	0	0
1928	3398del5	1	1	0	0
2043	4075delT	4	2	1	0
2362	6174delT	2	2	0	0
2371	6174delT	1	0	0	0
2317	IVS 2+2 T>C	1	1	0	0
2634	6174delT	1	0	0	0
2044	4706del4	17	6	2	0
2265	6174delT	2	2	0	0
2085	6503delTT	1	1	0	0
2349	6503delTT	2	0	1	0
2348	6503delTT	1	1	0	0
2306	IV14-2 A>G	2	1	0	0
2701	S611X	8	4	0	0
2367	IVS2+1 G>A	10	7	0	0
107	277delAC	16	10	1	0
Total		79	43	6	1

BC = breast cancer; OvC = ovarian cancer

Aim 3 (revised Statement Of Work of 2-6-96).
Analyze epidemiological and genetic risk factors in *BRCA2* mutation carriers.

During this past year, we participated in a series of collaborative studies to further characterize *BRCA1* and *BRCA2* mutation carriers.

A. In two studies, using a subset of our *BRCA1 and BRCA2* mutation carriers, we examined the response to radiation therapy and prognosis (Gaffney et al., 1998) and examined pathobiologic characteristics of hereditary breast cancer (Lynch et al., 1998). We found that there was no difference in prognosis between mutation carriers and breast cancer patients with no known germline (inherited) mutations (Gaffney et al., 1998), even though mutation carriers presented with tumors of higher nuclear grade, with increased

expression of DNA topoisomerase II-alpha, lacking hormone receptors, and more likely to have mutations in p53 (Lynch et al., 1998).

B. Hodgkin's disease patients who receive mantle irradiation have an age dependent increased risk of developing breast cancer. *BRCA2* interacts with RAD51, so it may be that *BRCA2* mutation carriers are more sensitive to radiation and therefore develop breast cancer after irradiation for treatment of Hodgkins lymphoma. Examination of loss of heterozygosity (LOH) was performed in order to give us an indication that a mutation in *BRCA2* or *BRCA1* might be involved, prior to expending the time and cost to screen for mutations. A case-control design was employed with case patients (previously treated with radiation therapy) matched with sporadic control breast cancer patients for age, breast cancer stage, and date of breast cancer diagnosis. After microdissection of tumor and normal tissue from paraffin-embedded tissue blocks, the extracted DNA samples were examined for LOH at chromosomal segments encompassing *BRCA1* and *BRCA2*. No statistical difference was observed between the case and control populations for LOH at *BRCA1* or *BRCA2*. In the Hodgkin's disease group, LOH was observed in 30% of tumors at *BRCA1* and 10% of tumors at *BRCA2* versus 10% and 0% of tumors in the control group at *BRCA1* and *BRCA2*, respectively. These data suggest that LOH at *BRCA1* and *BRCA2* is not involved in breast carcinogenesis in these patients.

For this next set of studies, we hypothesize that because breast (and ovarian) cancer is regulated by hormones, genetic and epidemiological factors which affect levels of circulating hormones may be important for risk of developing breast (ovarian) cancer.

C. In a collaborative case-control study of 207 women with ovarian cancer caused by *BRCA1* or *BRCA2* mutations and 161 of their sisters, we examined the effects of oral contraceptive use on risk of ovarian cancer (Narod et al., 1998). The adjusted odds ratio for ovarian cancer associated with any use of oral contraceptives was 0.5 (95% confidence interval, 0.3-0.8). The risk decreased with increasing duration of use; when used for six or more years there was a 60% reduction in risk.

D. In another collaborative study, we collected information on prophylactic oophorectomies and mastectomies from women with *BRCA1* and *BRCA2* mutations. We examined the effect of bilateral prophylactic oophorectomy (BPO) on breast cancer risk in *BRCA1* mutation carriers (at the time there were too few data for *BRCA2* mutation carriers)(Rebbeck et al., 1999). Previous population studies had reported that BPO reduces breast cancer risk in premenopausal women (20-50% depending on the study). The cohort consisted of 43 women with BPO and 79 women without BPO matched on clinic and year of birth ($\pm$ 5 years). Cox proportional hazards models were used to estimate differences in breast cancer incidence by BPO status. Age at menarche modified breast cancer risk so that the hazard ratios (HR) were adjusted for age at menarche. We found a statistically significant reduction in breast cancer risk after BPO with an adjusted hazard ratio of 0.53 (95% confidence interval = 0.33-0.84). Use of hormone replacement therapy did not negate the reduction in breast cancer risk.

E. In another study, we examined a common genetic variant which may modify age-specific penetrance in *BRCA1* mutation carriers. We hypothesized that because AR alleles of longer CAG repeats are associated with a decreased ability to activate androgen-responsive genes, they may increase the risk of breast cancer. We examined the association of the AR-CAG repeat length with breast cancer penetrance in a cohort of 304 women with germline *BRCA1* mutations, 165 with breast cancer and 139 without cancer (Rebbeck et al., 1999). Cox proportional hazards models were used to evaluate differences in age-specific penetrance across AR-CAG repeat lengths, both adjusted and unadjusted for hormone-related risk factors (age at menarche, age at first live birth, and parity). There was a significantly earlier age at diagnosis in women with at least one long AR-CAG ($\geq$ 28 repeats) than in those without one. Women who carried at least one AR allele with > 28, >29, >30 CAG repeats were diagnosed with breast cancer 0.8, 1.8, and 6.3 years earlier, respectively, than women who did not carry at least one such allele. This suggests that androgen signaling pathways may be important in modifying development of cancer, at least in *BRCA1*-associated cancers.

F. Other collaborative projects are ongoing. These include projects to investigate the association of variants in hormone synthesizing and metabolizing genes which may modify age-specific penetrance (age at diagnosis) and to determine if there is a reduction in risk of contralateral breast cancer following tamoxifen treatment for the first breast cancer. The data for the tamoxifen study are currently being analyzed by a collaborator, Dr. Narod, and a manuscript will be submitted.

Aim 4 (revised Statement Of Work of 2-6-96).
Perform haplotype analysis to study the origin of the *BRCA2* mutations. With the isolation of BRCA2, we were able to examine common mutations in order to identify whether they are founder mutations or areas of mutational hotspot. As described below, we performed many studies to identify and characterize founder mutations.

A. In collaboration with researchers at the University of Iceland, we found the 999del5 mutation in Icelandic breast cancer families and found that 16/21 (76%) carried the 999del5 mutation and all carried the same haplotype (Thorlacius et al., 1996).

B. We were the first to report on the founder *BRCA2* 6174delT mutation in Ashkenazi Jewish woman. We observed that 8% of Ashkenazi Jewish women with breast cancer diagnosed at < 50 years of age carry this mutation (Neuhausen et al., 1996). In a subsequent collaborative study, we determined that it is present in 1% of Ashkenazi Jews (Oddoux et al., 1996). In another collaborative study, we further explored the effects of three founder *BRCA1* and *BRCA2* mutations in Ashkenazi Jews. We examined families with two or more breast cancer cases with at least one case diagnosed at less than age 50 years (Tonin et al., 1996). Among the 220 eligible families, 82 also had cases of ovarian cancer. Twenty nine percent of the 138 families with only breast cancer had mutations in one of the three genes, and 73% of the 82 families with breast and ovarian cancer had mutations. In those families with only two cases of breast cancer and no ovarian cancer,

25% of families had a mutation. This suggests that if testing is to be offered, even small families should be eligible. We also screened for three other *BRCA2* mutations which had been identified in individuals of Ashkenazi Jewish descent, but none of them were observed in our cohort of 130 Ashkenazi Jewish women with breast cancer.

C. Founder populations descend from a relatively small group of individuals, and a mutation started in a founding individual will have been passed down through generations. During meiosis, the entire chromosome is passed down, except for when there is recombination. The longer the time since a mutation originated, the more opportunity for recombination so that the shared amount of DNA on a chromosome among mutation carriers will be less. Therefore, one can calculate how long ago these mutations arose, based on the amount of common DNA around the mutation. These are estimates with rather large confidence intervals, but they give an indication of how long ago they arose. One can then look historically at what was occurring at that time. We examined nine recurring *BRCA2* mutations from a total of 111 families in order to determine whether there was a common founder for each mutation, to estimate the age of the mutation, and to compare mutations to examine mutation specific phenotypes (i.e., an excess of ovarian cancer) (Neuhausen et al., 1998). Only the six mutations for which there were five or more haplotypes could be analyzed for age of the mutation. In total, we generated over 2,000 genotypes from 10 short tandem repeats in order to examine the 7 cM region surrounding the *BRCA2* gene. The 6174delT mutation which is frequent in Ashkenazi Jews (1.3%) was the most accurately estimated due to the large amount of data. We calculated the number of generations (G) since the mutation originated , with each generation approximated at 20 years. We estimated that the six mutations arose from 400-2,000 years ago (Table 2). There was no evidence for multiple origins of identical *BRCA2* mutations. The confidence limits are large, however, the 3034 del4 is clearly a much older mutation than the others. Our study data were consistent with the previous report of a higher incidence of ovarian cancer in families with mutations in a 3.3 kb region of exon 11 called the ovarian cancer cluster region (OCCR). When the age at diagnosis of the breast cancer cases was examined by OCCR, cases associated with mutations in the OCCR had a significantly older mean age at diagnosis than was seen in those outside this region (48 years versus 42 years).

Table 2. Common Haplotypes and Likely Age of Origin of Mutation

Mutation	# families	Age of mutation in years (1-Lod interval)
BRCA2-2034insA	5	720 (260-1280)
BRCA2-982del4	5	360 (80-860)
BRCA2-3034del4	11	1600 (920-2680)
BRCA2-6174delT	69	580 (440-760)
BRCA2-6503delTT	7	1040 (480-1960)

D. We continued to examine founder mutations (mutations occuring in specific populations), including the I1307K *APC* variant which was reported to occur in approximately 6% of Ashkenazi Jews. In a collaborative study, we examined the role of this mutation on occurrence of breast cancer in Ashkenazi Jewish women, by determining the frequency of this variant in a set of 632 women with primary invasive breast cancer and a set of 146 familial breast cancer cases (Redston et al., 1998). We concluded that the effect of the I1307K allele on breast cancer risk was largely limited to those with either *BRCA1* or *BRCA2* mutations.

Aim 5:

In April, 1997 we added this aim to examine other putative breast cancer susceptibility genes and regions in families for which no *BRCA1* or *BRCA2* linkage or mutations had been identified. We performed genotyping of 122 DNA samples from 11 high-risk breast cancer families which did not have *BRCA1* or *BRCA2* mutations. These 11 families were selected because their average expected LOD (ELOD) scores based on simulation analyses were all above 0.50 and therefore sufficient to detect linkage. The maximum ELODs for each kindred ranged from 1.1 to 3.2. The candidate chromosomal regions to be analyzed for linkage include 8p, 11p15, 11q13 and 11q22, 15q14, 16q22-24, 18q11-13, 20q13, 22, and the candidate genes include the progesterone receptor, BCSC1, DAB2, e-cadherin, and PTEN/MMAC.

Table 3. Families being examined for other breast cancer susceptibility genes

Kindred	Total # cases	DNA from cases	Diagnosis age range in years
1927	5	3	42-60
1929	7	3	33-72
2019	10	4	43-79
2036	5	3	34-60
2260	5	4	31-50
2262	6	5	48-53
2308	9	4	36-71
2324	6	3	38-81
2329	15	5	29-90
2370	3	3	39-64
2381	6	4	41-70

There was no evidence for linkage to any of the candidate regions or loci. LOD scores ranged from 0.00 to 1.034, none of which meet the criteria for suggestive evidence of linkage. Unlike *BRCA1* and *BRCA2*, where there are specific phenotypes associated with each gene, e.g. ovarian cancer and breast cancer for *BRCA1* and male and female breast cancers for *BRCA2*, no phenotype with which to stratify families has been identified. It may be that localization of

additional breast cancer genes will need to await more knowledge of genes which interact with *BRCA1* and *BRCA2* or a larger set of families.

KEY RESEARCH ACCOMPLISHMENTS:

- Isolation of *BRCA2*
- Identification of the founder *BRCA2* 6174delT mutation in Ashkenazi Jews
- Identification of the founder *BRCA2* 999del5 mutation in Icelanders
- Evaluation of the contribution of *BRCA1* and *BRCA2* to breast cancer in high-risk breast cancer families
- Investigation of ages of recurrent, founder mutations
- Determination of age-specific penetrance and risks of other cancers for *BRCA2* mutation carriers
- Assessment of the effect of oral contraceptive use in reducing risk of ovarian cancer in *BRCA1* and *BRCA2* mutation carriers
- Investigation of the effect of bilateral prophylactic oophorectomy in reducing risk of breast cancer in *BRCA1* mutation carriers
- Examination of the association of androgen receptor CAG repeat length with penentrance of breast cancer in mutation carriers

REPORTABLE OUTCOMES:

A. Manuscripts.

1995

Goldgar DE, Neuhausen SL, Ward JH, Steele L, Fields P, Tran TD, Nguyen K, Stratton MR, Easton DF: (1995) A 45-year follow-up of kindred 107 and the search for BRCA2. J Natl Cancer Inst., 17:15-19.

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C. Presentations:

Neuhausen S.L. May 1997. Genetics of Breast Cancer Genes BRCA1 and BRCA2. University of Chicago Hematology and Oncology Seminar Series.

Neuhausen SL. September, 1997. Founder effects in BRCA2. Breast Cancer Linkage Consortium.

Neuhausen S. L. June 1998. Ethnic differences in cancer risk. American Cancer Society. Second National Conference in Cancer Genetics: Pragmatic aspects and implications for clinical practice.

Neuhausen, S. L. September 1999. Fred Hutchinson Cancer Center Genetics Lecture, Genetic Epidemiology of BRCA1 and BRCA2.

D. Funding applied for based on work supported by this award:

Awarded

RPG-99-181-01-CCE (Susan L. Neuhausen) 07/01/99 to 06/31/02

American Cancer Society $111,200

Biomarkers of Cancer Risk for *BRCA1* and *BRCA2* Mutation Carriers

Goals: To identify modifier genes which affect overall incidence and age of onset of breast and ovarian cancers in a set of *BRCA1* and *BRCA2* mutation carriers. The genes to be examined are involved in hormone synthesis and metabolism or in metabolism of environmental carcinogens.

Pending

(Susan L. Neuhausen) 04/01/00 to 03/31/03

U.S. Army $75,000

An investigation of genes which may modify cancer risk in carriers of *BRCA1* and *BRCA2* mutations

CONCLUSIONS:

The primary objective of this grant was to clone *BRCA2* and it was achieved. With the cloning of *BRCA1* and *BRCA2*, two genes causing inherited susceptibility to breast and ovarian cancers, studies focused on understanding the etiology of breast cancer could be undertaken. The hope is that this knowledge can be used to reduce the incidence of breast cancer and/or breast cancer mortality. In addition, it is now possible to predict risk in a subset of women who carry mutations in these genes.

Other important findings from this grant include identifying the founder mutations, 6174delT in Ashkenazi Jews and 999del5 in Icelanders, investigating origins of recurrent mutations, identifying mutations and mutation carriers for studies of penetrance and risks of other cancers, and examining the effect of oral contraceptive use on reducing risk of ovarian cancer and the effect of bilateral prophylactic oophorectomy on reducing risk of breast cancer. Funding from this grant resulted in 24 publications including 20 research articles, 3 review articles, and 1 book chapter.

We observed that individuals of Ashkenazi Jewish descent have an increased risk of breast cancer from three founder mutations in *BRCA1* and *BRCA2* . This suggested that genetic testing can be stratified to first test for these three recurrent mutations which account for approximately 20-30% of breast cancer in this population. There are recurrent mutations in other populations

which may be important for risk assessment including the 999del5 mutation in Icelanders, the 4484delG in the Swedes, and the 5573insA in the Dutch.

From our collaborative studies with the BCLC, better estimates of age-specific penetrance and risks of other cancers are now available and can be used in clinical risk assessment.
It is clear that not all women who carry deleterious mutations in *BRCA1* and *BRCA2* will develop breast cancer and that the age at which it develops is variable. There are other factors which modulate risk and we have explored some of those with funding from this grant. Results from studies of phenotype-genotype correlations; of breast cancer risk associated with hormonal factors including variants in hormone-metabolizing genes, oral contraceptives, and prophylactic surgeries; and of prognostic indicators will hopefully be used to target specific areas of *BRCA2* for basic biology research. Better understanding of *BRCA2* should allow for development of better therapeutics and preventative measures, as well as for more accurate individual cancer risk assessment for women at high risk to develop cancer.

REFERENCES.

See manuscripts listed in the "Reportable Outcomes" section (Page 13).

APPENDIX.

Copies of all publications supported by U S Army Grant DAMD 17-94-J-4260.

FINAL REPORT.

A. For bibliography of all publications and meeting abstracts, see "Reportable Outcomes" section (Page 13).

B. Personnel who received pay from the research effort:

NAME	POSITION TITLE
Abtin, Vicki R.	Research Coordinator
Albright, Lisa A.	Ph.D. , Investigator
Anderson, Michelle C.	Research Coordinator
Armstrong, Dustin A.	Lab Aide
Ashton, Michael B.	Computer Technician
Bansal, Aruna	Ph.D., Investigator
Bayley, Heather D.	Clerk
Black, Jeff	Research Coordinator
Christensen, Chad T.	Phlebotomist
Geisler, Jean	Technical Writer
Goldgar, David E.	Ph.D., Investigator
Hanson, Janice K.	Staff Research Assistant
Hedgepeth, Adam M.	Clerical Assistant
Jacobsen, Matthew S.	Computer Technician

Kort, Edward N.	Ph.D., Research Associate
Lewis, Cathryn M.	Ph.D., Investigator
Melendez, Gonzalo J.	Computer Professional
Mitchell, Jared D.	Technical Assistant
Neuhausen, Susan L.	Ph.D., Investigator
Nguyen, Kim N.	Lab Specialist
Platts, Christopher	Lab Aide
Steele, Linda H.	Research Coordinator
Swensen, Jeff J.	Research Assistant
Tran, Phuong Tuyet	Lab Assistant
Tran, Thao Dinh	Lab Specialist
Tran, Hien Dinh	Lab Technician
Yakumo, Kazuko	Lab Technician

APPENDIX

PUBLICATIONS SUPPORTED BY USAMRMC U S ARMY GRANT DAMD 17-94-J-4260

"Identification and Genetic Mapping of Genes for Hereditary Breast and Ovarian Cancer in Families Unlinked to BRCA1"

Manuscripts
Abstracts

A 45-Year Follow-up of Kindred 107 and the Search for BRCA2

*David E. Goldgar, Susan L. Neuhausen, Linda Steele, Patty Fields, John H. Ward, Thao Tran, Kim Ngyuen, Michael R. Stratton, Douglas F. Easton**

Interest in the genetics of breast cancer has intensified with the discovery of a breast cancer susceptibility locus, BRCA1, on chromosome 17q. In this paper, we describe updated information on a large breast cancer kindred (K107) that has been extensively studied since 1948. Specifically, we have identified many new cases of cancer in the family and have shown that this family is unlinked to BRCA1 as well as a number of other genes considered as candidates for breast cancer. In a collaborative study between the University of Utah and the Institute of Cancer Research in the United Kingdom, we have collected a set of families with a predisposition to breast and ovarian cancers that have been reliably excluded from linkage to BRCA1 and evaluated their usage in a genomic search for other breast cancer loci. This effort led to the discovery of a second breast cancer locus located on chromosome 13q, BRCA2, which is responsible for the increased incidence of breast cancer in Kindred 107. [Monogr Natl Cancer Inst 17:15-19, 1995]

Although a state with a relatively small population base, Utah has proven to be an excellent location for studying genetic predisposition to common disease. This is due, in part, to polygamy practiced among the Utah pioneers and to the emphasis of the Church of Jesus Christ of Latter-day Saints ("Mormon") on large families and the maintenance of genealogical records. This genealogical resource has been linked to the Utah Cancer Registry (UCR) and other public records to form the Utah Population Database. This resource facilitates the identification and analysis of families with apparent genetic predisposition to cancer. One such family, Kindred 2082, contains 35 cases of breast or ovarian cancer due to the BRCA1 gene and has been recently studied in detail (*1*). The focus of this paper, however, is on families whose predisposition to breast cancer is not due to BRCA1. In particular, we will present an update on one such unlinked kindred (K107), which contains 47 cases of breast or ovarian cancer, and discuss our strategy for localizing additional genes that confer susceptibility to breast cancer.

Perhaps the earliest large kindred with an apparent inherited susceptibility to breast cancer was that reported by Gardner and Stephens (*2*) in 1950 and identified as Kindred 107 (K107). This family was originally ascertained in 1947 by a University of Utah genetics student with two great-aunts who died of breast cancer in their 40s. Subsequent clinical and genealogical follow-up identified seven additional cases of breast cancer and many benign breast tumors. The family was updated several times, most notably in 1980 (*3*). In a study by Bishop and Gardner (*3*), 22 new cases of breast and ovarian cancers were identified and the penetrance of the gene was estimated to be 0.82 by age 80. More recently, we have attempted to update the cancer status of all members of the kindred and to explore cancer incidence in new branches of the family. This task was somewhat complicated because, although much of the family remains in the Salt Lake valley, parts of the family are scattered across the western United States and Canada. More important, attempts were made to obtain blood samples from a large number of affected women and their relatives in K107. The kindred is now known to contain 38 cases of female breast cancer, three cases of male breast cancer, and six cases of ovarian cancer. Fig. 1 shows a reduced pedigree drawing of K107 showing the temporal identification of cases, while Table 1 shows the distribution of age at diagnosis of the breast and ovarian cancer cases in K107. We were able to obtain histology coding from the UCR or from medical records for 18 of the cases shown in Table 1 and Fig. 1. Of these cases, 11 were coded as infiltrating ductal, four were adenocarcinoma (not otherwise specified), one was intraductal papillary carcinoma, and one was a case of Paget's disease.

Although at this stage we have not formally re-estimated the penetrance of the gene responsible for the breast cancer in K107, examination of the status of obligate carriers (i.e., connecting parents of cases) in Fig. 1 demonstrates that the hypothesized locus is highly penetrant. The phenotypic status of obligate carriers is shown in Table 2. As one would expect, the vast majority of the female obligate carriers were affected with cancer or benign premalignant lesions; however, it is noteworthy that eight of the 17 male obligate carriers who survived until at least age 55 developed a malignancy. Conspicuously, four obligate carriers developed prostate cancer at ages ranging from 55 to 80 years. A number of other cancers appear to be associated with the expression of this gene, most notably prostate

**Affiliations of authors:* D. E. Goldgar, S. L. Neuhausen, L. Steele, P. Fields, T. Tran, K. Ngyuen (Department of Medical Informatics), J. H. Ward (Division of Hematology-Oncology, Department of Medicine), University of Utah, Salt Lake City.

M. R. Stratton, D. F. Easton, Institute of Cancer Research, Sutton, United Kingdom.

Correspondence to: David E. Goldgar, Ph.D., Genetic Epidemiology, 420 Chipeta Way, Suite 180, Salt Lake City, UT 84108.

See "Notes" section following "References."

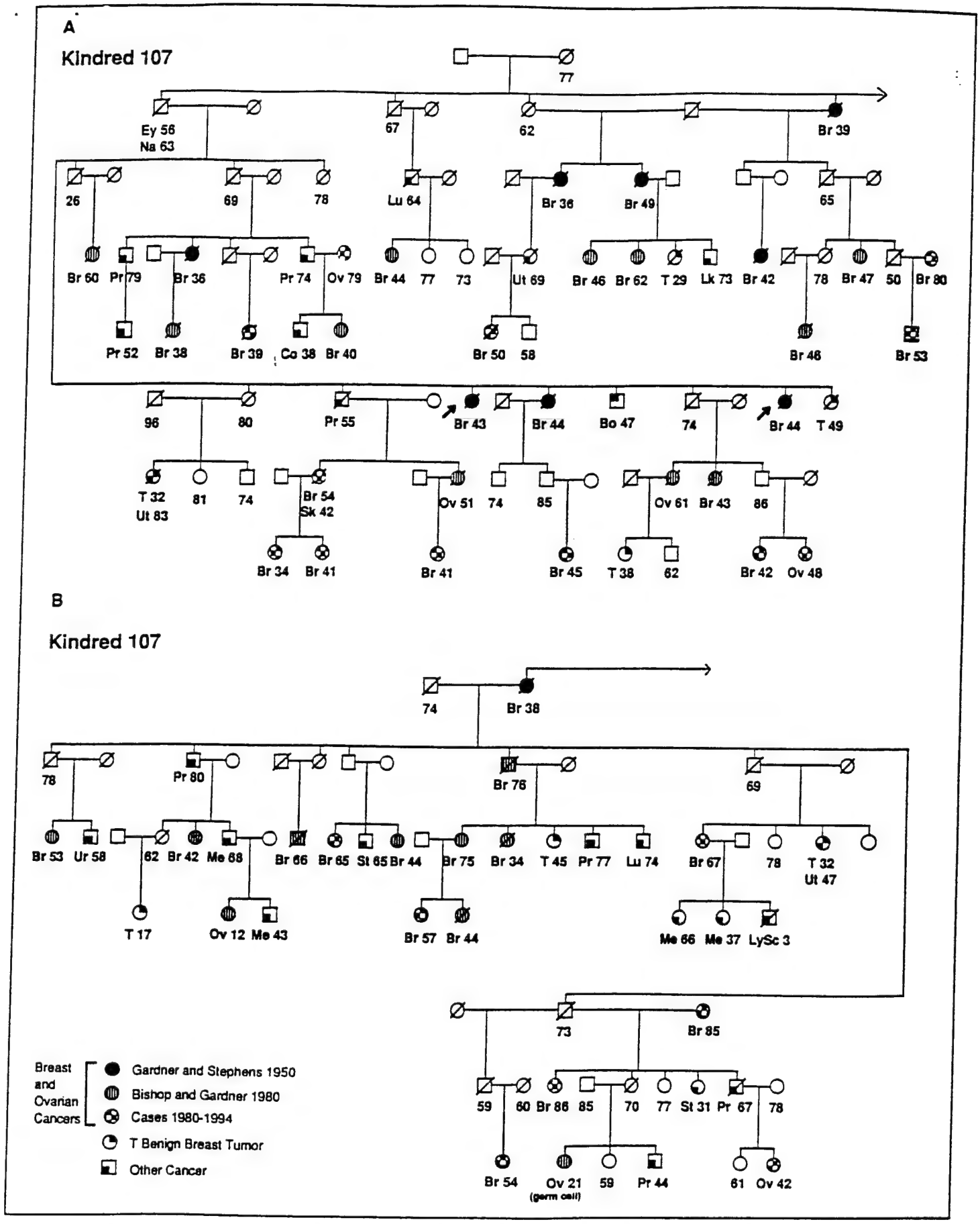

Fig. 1. A and B. Pedigree diagram of K107 showing the relationships between all cases of breast or ovarian cancer identified to date in K107. Totally filled symbols represent cases of breast cancer that were described in the original report of Gardner and Stephens (2); symbols denoted with vertical striping are those additional cases that appeared in the update of Bishop and Gardner (3); and symbols with the black and white hatched pattern were identified in the present study. Symbols with shading in the upper right-hand quarter are women who were reported to have had benign breast tumors on the basis of histology of biopsied material. Lower left quarter shading represents cancer at sites other than breast or ovarian cancer.

Table 1. Age at diagnosis of K107 breast and ovarian cancers*

Cancer	Age at diagnosis, y			
	≤40	40–49	50–59	≥60
Breast	8	17	7, M	6, M, M
Ovarian	2	2	1	1

*M = male breast cancer.

Table 2. Cancers at other sites in relatives of breast and ovarian cancer cases*

Site or type	First-degree relatives		Second- and third-degree relatives	
	No.	Ages at diagnosis, y	No.	Ages at diagnosis, y
Prostate	6	44, 55,† 67,† 74, 75,† 80†	1	52
Uterus	3	44, 70,† 82‡	1	47‡
Melanoma	2	43, 72	3	37, 66, 68†
Navel	1	63†		
Eye	1	56†		
Bone	1	47	1	7§
Colon	1	38	1	68§
Urethra	1	61		
Basal cell	1	41‡		
Lip			1	64§
Lymphoma	1	56		
Lung	2	64,† 74		
Leukemia	1	73		
Lymphosarcoma			2	3, 9§
Osteosarcoma			1	12§
Throat			1	49§
Stomach			1	31
Thyroid			1	9§

*Only relatives of breast cancer patients diagnosed under age 60, male breast cancer, or ovarian cancer are listed.
†Cancer found in an obligate carrier of a presumed susceptibility locus.
‡Second cancer in a woman with breast or ovarian cancer.
§Third-degree relative; all others are second-degree.

cancer, melanoma, and uterine cancer, as shown by both the obligate carriers and in the cancers found in relatives of affected women in the family (Table 2). Interestingly, population-based studies of familial cancer have shown possible familial associations between breast and prostate cancers (*4*) and breast, prostate, and uterine cancers (*5,6*).

After obtaining a sufficient number of DNA samples, studies were undertaken to identify the gene responsible for the unusual cluster of breast cancer cases in K107. A natural place to start such a search, of course, is the BRCA1 gene for familial breast and ovarian cancers on chromosome 17q (*7-9*).

Linkage to the BRCA1 region was investigated in this kindred by genotyping four markers that cover the region known to contain BRCA1. Linkage to BRCA1 could be reliably excluded in K107 based on the analysis of the 17q marker typings. In addition to the negative logarithm of the odds (LOD) scores with 17q markers shown in Table 3, at least 10 distinct 17q BRCA1-region haplotypes were observed among breast cancer patients diagnosed under age 50, male breast cancer, or ovarian cancer. In addition, a series of other loci that could be considered to be candidate regions or candidate genes for breast cancer were tested and similarly excluded (Table 3). These included regions defined by loss of heterozygosity in breast or ovarian tumors, loci involved in familial cancer at other sites, or loci for which some evidence of involvement in familial breast cancer has been previously reported.

The phenotypic pattern of cancer observed in K107 does not appear to be an isolated occurrence. A systematic survey was undertaken to identify all families containing at least one case of male breast cancer, at least two cases of female breast cancer diagnosed under the age of 50, and one or more cases of ovarian cancer (designated "BMO" families). In collaboration with M. Stratton at the Institute of Cancer Research (ICR), a set of 12 such families were collected together with 10 families that met the above description but did not have any cases of ovarian cancer (*10*). Based on genotypings for markers surrounding BRCA1, no evidence for BRCA1 linkage was found. The best estimate of the proportion of families linked was zero, with an upper 95% confidence limit of 20%.

To localize the breast cancer susceptibility locus in these families, as well as in other breast cancer families unlinked to BRCA1, a collaboration was formed between the ICR and the University of Utah. Initially, a set of 22 families, consisting of five of the most informative of the BMO families, five unlinked breast and ovarian cancer families without a male case, and 12 families with early-onset female breast cancer only were examined to determine their power for localizing additional breast cancer loci under a variety of situations. As detailed below, simulation studies using the program SLINK (*11*) showed this set to contain sufficient power to localize one or more breast cancer susceptibility loci, even if further genetic heterogeneity is present.

Assumed Model for Candidate Gene Tests

Since in this case we are testing a specific-candidate gene, we assumed in the simulations that we had a polymorphic marker

Table 3. Linkage analysis of chromosome 17q markers and candidate regions in K107

Chromosome 17q markers		LOD scores	
Locus		θ = 0.001	θ = 0.05
THRA1		−2.22	−0.47
D17S855		−3.03	−1.55
D17S1325		−2.43	−1.70
D17S1184		−3.57	−2.14
Gene/region	Candidate locus for breast and ovarian cancers		LOD (θ = 0.01)
p53	D17S796		−2.47
Estrogen receptor	ER6		−2.55
CAR/16q	D16S413		−2.99
MTS1/MLM	CT29		−3.37
MSH2	D2S123		−1.55
Mlh1	D3S1289		−1.58

within the gene complex of interest or, alternatively, close-flanking markers surrounding the gene. Accordingly, we assumed for these simulations a recombination fraction of 0.001 between the disease and a marker locus assumed to have five equally frequent alleles. The model for the disease gene was assumed to be that described in a study by Easton et al. (*9*). Because it is unlikely that any single-candidate gene would be responsible for all non-BRCA1 familial breast cancer, we also conducted the simulations under an assumption that, on average, 50% of the families would be linked to a given candidate locus.

Assumed Model for Genomic Search

In this case, we assume that we have flanking markers each located 10 cM (centimorgan) from the hypothesized disease locus. To ensure that an adequate number of replicates could be simulated in a reasonable amount of computer time, we modeled two flanking 4-allele markers as a single locus with 10 equally frequent alleles, located 9 cM from the disease. On small pedigrees, these two models have roughly equivalent information content: we assumed that this was also the case for the larger pedigrees. Again, we simulated linkage homogeneity and 50% heterogeneity.

The studies described above demonstrated that our set of families have more than adequate power if there is only minimal further heterogeneity (in addition to BRCA1) or if the genetic heterogeneity is accounted for largely by our phenotypic classification. It seems likely that the BMO classification is a genetically homogeneous subset. If heterogeneity is present throughout the family material and is of the order of 50%, we have shown that we still have a reasonable chance (65%) of detecting linkage to a breast cancer susceptibility locus. If the degree of genetic heterogeneity is higher, it may still be possible to detect linkage to one or more susceptibility loci, since we have several individual families in our set, each of which is capable of achieving a LOD score greater than 3.0.

The Search for BRCA2

As a first step in identifying additional breast cancer susceptibility loci, linkage between breast cancer and the candidate loci shown in Table 3 was tested in a combined set of 15 families. Each of the candidate loci could be excluded from playing a significant role in the breast or ovarian cancer in these families. Initially, 200 markers spaced at roughly 20-30 cM across the genome were examined for linkage in the University of Utah and ICR families. When no significant evidence of linkage was found to any of these initial markers, a second set of markers from the newer Genethon set (*12*) was used to fill in the gaps in the previous set and to cover areas of the genome that were not excluded. During the course of this latter analysis, a hint of linkage was found to D13S260 in the ICR families, and this was subsequently confirmed in the University of Utah set of families. In all, 15 families were analyzed for five markers on chromosome 13q. A maximum multipoint LOD score was found between breast and ovarian cancers and D13S260/D13S67 of 9.58, with significant evidence of genetic heterogeneity (*13*). This locus was denoted BRCA2. An estimated 74% of the families were linked to BRCA2; the LOD score under heterogeneity was 11.65. Among the families that showed convincing evidence of linkage to 13q was K107, which had a multipoint LOD score of 3.48 (*13*). Interestingly, although K107 did have six cases of ovarian cancer, four of these cases did not carry the linked 13q haplotype. Thus, the role of BRCA2 in ovarian cancer remains uncertain.

Conclusion

K107 may be representative of a new familial cancer syndrome consisting of early-onset female breast cancer, ovarian cancer, at least one case of male breast cancer, and, possibly, prostate cancer, uterine cancer, and melanoma. Linkage analysis has shown that the gene responsible for K107 and the majority of families with a similar phenotype is located on chromosome 13q. Prospective follow-up of K107 will continue to provide useful information on the penetrance of the susceptibility locus and its associations with cancers at other sites. This effort will now be facilitated by the presence of a linked haplotype that will allow more precise estimation of the effects of the BRCA2 mutation segregating in K107. Although most breast cancer and breast and ovarian cancer families appear to be due to either BRCA1 or BRCA2, a number of high-penetrant, early-onset families are inconsistent with linkage to both BRCA1 and BRCA2, indicating the presence of one or more additional breast cancer loci. The collaboration between the University of Utah and the ICR is continuing to search the genome, with the goal of identifying these additional loci.

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Notes

Supported in part by USARMC grant DAMD17-94-J-4260; and by Public Health Service grants CA48711 (National Cancer Institute), HG00571 (National Center for Human Genome Research), and CN05222 (Division of Cancer Prevention and Control, National Cancer Institute), National Institutes of Health, Department of Health and Human Services.

We acknowledge the contribution of all of the individuals involved in earlier studies of Kindred 107, particularly Dr. Eldon Gardner.

(GSK3-α), was the site of phosphorylation in each phosphopeptide, both *in vitro* (Fig. 4*b*) and *in vivo* (not shown). The ^{32}P-labelling of other (more acidic) tryptic phosphopeptides was not increased by insulin (Fig. 4*d*). These peptides have been noted previously in GSK3 from A431 cells and shown to contain phosphoserine and phosphotyrosine[11].

PKC-δ, ε and ζ are reported to be activated by mitogens, and PKC-ζ activity is stimulated *in vitro* by several inositol phospholipids, including PI(3,4,5)P_3, the product of the PI 3-kinase reaction[26]. However, purified PKC-ε[27], PKC-δ and PKC-ζ (data not shown) all failed to inhibit GSK3-α or GSK3-β *in vitro*. Moreover, although PKC-α, β1 and γ inhibit GSK3-β *in vitro*[?], GSK3-α is unaffected, while their downregulation in L6 myotubes by prolonged incubation with phorbol esters abolishes the activation of MAPKAP kinase-1 in response to subsequent challenge with phorbol esters, but has no effect on the inhibition of GSK3 by insulin (not shown).

Taken together, our results identify GSK3 as the first physiologically relevant substrate for PKB. The stimulation of glycogen synthesis by insulin in skeletal muscle involves the dephosphorylation of Ser residues in glycogen synthase that are phosphorylated by GSK3 *in vitro*[2]. Hence the 40–50% inhibition of GSK3 by insulin, coupled with a similar activation of the relevant glycogen synthase phosphatase[28], can account for the stimulation of glycogen synthase by insulin in skeletal muscle[2] or L6 myotubes[29]. The activation of glycogen synthase and the resulting stimulation of glycogen synthesis by insulin in L6 myotubes is blocked by wortmannin, but not by PD 98059 (ref. 29), just like the activation of PKB and inhibition of GSK3. However, GSK3 is unlikely to be the only substrate of PKB *in vivo*, and identifying other physiologically relevant substrates will be important because PKB-β is amplified and overexpressed in many ovarian neoplasms[23]. □

Received 4 August; accepted 14 November 1995.

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ACKNOWLEDGEMENTS. We thank A. Klip for providing L6 cells, and H. McDowell for advice and help in growing them; A. Saltiel for PD 98059; J. Vandenheede for anti-GSK3 antibodies; C. Marshall for anti-p42 MAP kinase antibodies; P. Parker for PKC-δ and C. N. Morrice for providing GSK3; B. Caudwell for peptide sequencing; M. Frech for the PH domain antibodies, and J. Thorner for critical reading of the manuscript. D.A.E.C. is the recipient of a CASE studentship from the UK Biotechnology and Biological Sciences Research Council and SmithKline Beecham Pharmaceuticals. This work was supported by the British Diabetic Association, the UK Medical Research Council and The Royal Society.

Identification of the breast cancer susceptibility gene *BRCA2*

Richard Wooster*, Graham Bignell*, Jonathan Lancaster‡, Sally Swift†, Sheila Seal*, Jonathan Mangion*, Nadine Collins*, Simon Gregory§, Curtis Gumbs∥, Gos Micklem§, Rita Barfoot*, Rifat Hamoudi*, Sandeep Patel*, Catherine Rice§, Patrick Biggs*, Yasmin Hashim*, Amanda Smith†, Frances Connor†, Adalgeir Arason¶, Julius Gudmundsson¶, David Ficenec*, David Kelsell=, Deborah Ford**, Patricia Tonin**, D. Timothy Bishop††, Nigel K. Spurr=, Bruce A. J. Ponder‡‡, Rosalind Eeles*, Julian Peto**, Peter Devilee§§, Cees Cornelisse§§, Henry Lynch∥∥, Steven Narod**¶¶, Gilbert Lenoir¶¶, Valdgardur Egilsson¶, Rosa Bjork Barkadottir¶, Douglas F. Easton==, David R. Bentley§, P. Andrew Futreal∥, Alan Ashworth† & Michael R. Stratton***

Sections of * Molecular Carcinogenesis and ** Epidemiology, and † CRC Centre for Cell and Molecular Biology, Institute of Cancer Research, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK, and Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK
‡ Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA
§ The Sanger Centre, Hinxton Hall, Hinxton, Cambridgeshire CB10 1RQ, UK
∥ Duke University Medical Centre, Departments of Surgery and Genetics, and Division of Gynaecologic Oncology, Research Drive, Medical Sciences Research Building, Room 363, Durham, North Carolina 27710, USA
¶ Laboratory of Cell Biology, University Hospital of Iceland, P.O. Box 1465, IS-121 Reykjavik, Iceland
= ICRF Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar EN6 3LD, UK
** Division of Medical Genetics and Division of Human Genetics, Dept of Medicine, McGill University, 1650 Cedar Avenue, Montreal, H3G 1A4, Canada
†† ICRF Genetic Epidemiology Laboratory, 3K Springfield House, Hyde Terrace, Leeds LS2 9LU, UK
‡‡ CRC Human Cancer Genetics Research Group, Level 3, Laboratories Block, Box 238, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ, UK
§§ Department of Human Genetics and Pathology, Leiden University, Wassenaarseweg 72, P. O. Box 9503, 2300 RA, Leiden, The Netherlands
∥∥ Department of Preventive Medicine and Public Health, Creighton University School of Medicine, Omaha, Nebraska 68178, USA
¶¶ International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69372 Lyon Cedex 08, France
== CRC Genetic Epidemiology Group, Department of Community Medicine, Institute of Public Health, University of Cambridge, University Forvie Site, Robinson Way, Cambridge CB2 2SR, UK
** Women's College Hospital, Toronto, Ontario, Canada
*** Genome Sequencing Centre, Washington University in St Louis, School of Medicine, St Louis, MO, USA

IN Western Europe and the United States approximately 1 in 12 women develop breast cancer. A small proportion of breast cancer cases, in particular those arising at a young age, are attributable to a highly penetrant, autosomal dominant predisposition to the disease. The breast cancer susceptibility gene, *BRCA2*, was recently localized to chromosome 13q12-q13. Here we report the identification of a gene in which we have detected six different germline mutations in breast cancer families that are likely to be due to *BRCA2*. Each mutation causes serious disruption to the open reading frame of the transcriptional unit. The results indicate that this is the *BRCA2* gene.

10,4?? bp; -3.75kd

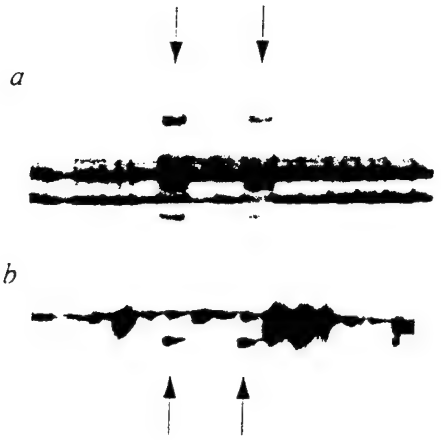

FIG. 1 Detection of the *BRCA2* gene mutation in family IARC 2932. Mutation screening by migration shift assays. The arrows indicate abnormally migrating bands in two early onset breast cancer cases from IARC 2932.
METHODS. A ^{32}P-labelled, 271-bp genomic fragment was amplified from lymphocyte DNAs from affected individuals in 46 breast cancer families. The PCR product was denatured in 50% formamide and electrophoresed through *a*, 4.5% non-denaturing polyacrylamide gels and *b*, 6% denaturing polyacrylamide gels.

Abnormalities of several genes are known to confer susceptibility to breast cancer. The *BRCA1* gene accounts for the large majority of families with both breast and ovarian cancer cases, but only half of families with site-specific breast cancer[1]. Using families with multiple cases of early-onset breast cancer showing evidence against linkage to *BRCA1* we recently demonstrated the existence of a second major breast cancer susceptibility locus, *BRCA2*, on chromosome 13q12-q13 (ref. 2). Preliminary studies indicate that mutations in *BRCA2* confer a similar risk of female breast cancer to *BRCA1*. However, the risk of ovarian cancer appears to be lower and the risk of male breast cancer substantially higher. Risks of other cancers, including prostate and laryngeal cancer, may also be elevated in carriers of *BRCA2* mutations (unpublished data).

BRCA2 was originally positioned within a 6-cM region between *D13S289* and *D13S267* that was defined on the basis of meiotic recombinants in early-onset breast cancer cases within clearly linked families[2]. (The genetic map in this region is centromere–*D13S289*–3cm–*D13S260*–1cM–*D13S171*–2cM–*D13S267*–telomere[3].) We further mapped the centromeric boundary of the interval within which the gene lies to *D13S260* using a set of Icelandic families (unpublished data). Subsequently, using recombinants in other families and additional microsatellite markers isolated from the region, we established that *BRCA2* is likely to be located in a 600-kb interval centred around *D13S171*. An unexpected contribution to the fine localization of *BRCA2* was provided by the detection of a homozygous somatic deletion in a single pancreatic cancer[4]. The centromeric boundary of this deletion is approximately 300 kb centromeric to *D13S171* and the telomeric boundary close to, but still centromeric of, *D13S171* (ref. 5). Despite the ambiguity of the relationship between this deletion and *BRCA2*, we combined the genetic recombinant information from families and the physical localization from the homozygous deletion, and prioritized analysis of the 300-kb region immediately centromeric to *D13S171*.

Yeast artificial chromosome (YAC)[6] and P1 artificial chromosome (PAC)[7] contigs extending approximately 700 kb centromeric and 300 kb telomeric to *D13S171* were constructed and a minimally overlapping set of 14 PACs was identified. Transcribed sequences located on these genomic contigs were identified using two methods: exon amplification (exon trapping) from subcloned PAC DNA[8], and direct selection by solution hybridization of complementary DNA to PAC genomic DNA[9]. To identify *BRCA2*, genomic DNA fragments of less than 300 bp containing putative coding sequences were screened for mutations. At least one affected member of 46 breast cancer families was examined. Each family included in this set either shows evidence of linkage to *BRCA2*, and or shows evidence against linkage to *BRCA1*, and or has not been found to carry a *BRCA1* mutation, and or includes a case of male breast cancer. Most, but probably not all, of these families would be expected to have cases caused by *BRCA2* mutations.

Disease-associated mutations in most known cancer susceptibility genes usually result in truncation of the encoded protein and inactivation of critical functions. In the course of the mutational screen of candidate coding sequences from the *BRCA2* region, the first detected sequence variant that was predicted to disrupt translation of an encoded protein was observed in IARC 2932 (Fig. 1). This family is clearly linked to *BRCA2* with a multipoint LOD score of 3.01 using *D13S260* and *D13S267*. A deletion of 6 bp removes the last five bases of the exon examined (exon S66), deletes the conserved G of the 5′ splice site of the intron, and directly converts the codon TTT for phenylalanine to the termination codon TAA. By sequencing, this mutation has been detected in lymphocyte DNA from two other early-onset breast cancer cases in this family. The individuals examined share only the disease-associated haplotype. The mutation is absent in more than 500 chromosomes from normal individuals and in the remaining families and cancers. This finding therefore identified a strong candidate for the *BRCA2* gene.

TABLE 1 *BRCA2* mutations in breast cancer families

	FBCs	FBCs < 50	OvCs	MBCs	LOD score at *BRCA1*	LOD score at *BRCA2*	*BRCA2* mutation
IARC 2932	15	10	0	0	−2.38	3.01	CCC.TTT.CGgtaa
IARC 3594	6	5	0	0	nd	nd	CAT.AAC.TCT.CTA
CRC B211	5	3	4	0	−0.48	0.49	AGT.CTT.CAC
CRC B196	17	12	0	0	−2.21	0.92	AAA.ACT.GAA.ACT
Montreal 681	3	2	0	1	nd	nd	GCA.AGT.GGA
Montreal 440	2	2	0	2	nd	nd	GAT.AAA.CAA.GCA

LOD scores at *BRCA1* were calculated using the markers *D17S250* and *D17S579*; those at *BRCA2* were calculated using the markers *D13S260* and *D13S267*. Exon sequence is denoted by upper case, intron sequence by lower case; Codons are indicated by stops. The underlined letters indicate the deleted bases in each family. Abbreviations: FBCs, female breast cancers; OvCs, ovarian cancers; MBCs, male breast cancers.

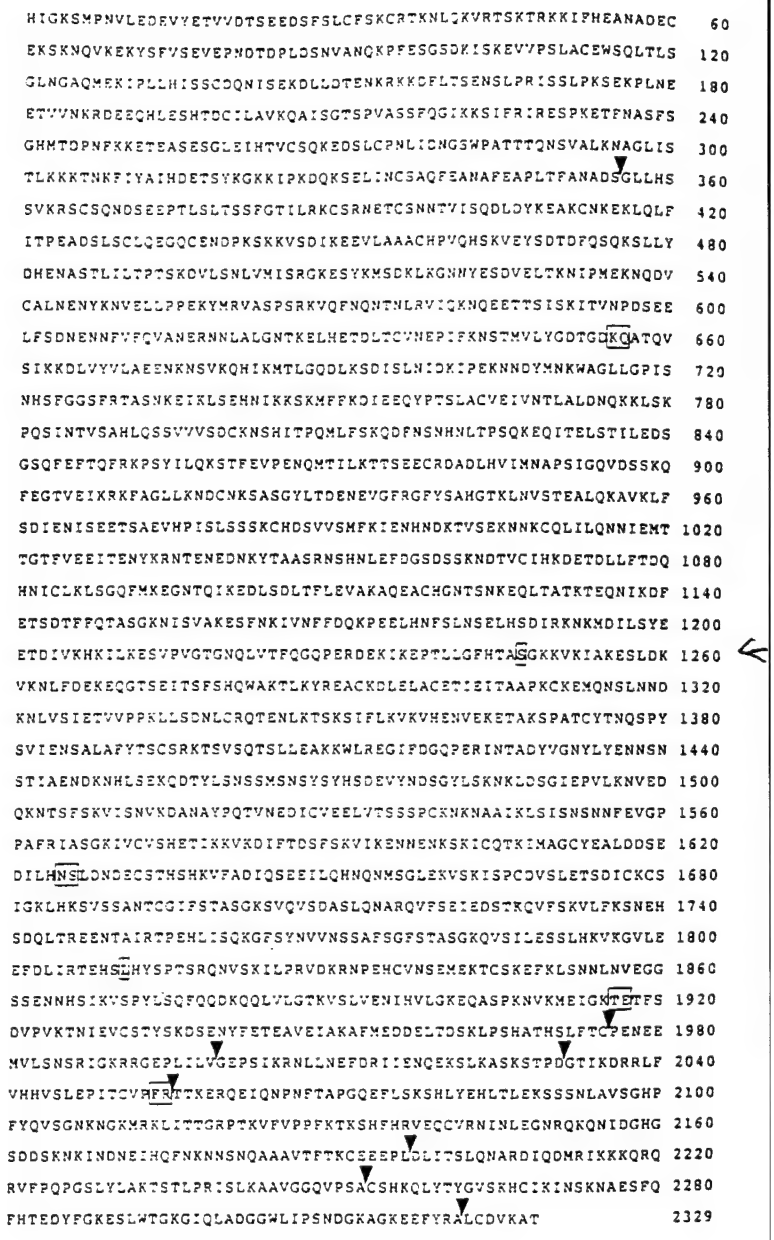

FIG. 2 Predicted amino acid sequence of the *BRCA2* gene. The positions of the frameshift mutations indicated in Table 1 are boxed, and the positions of intron–exon boundaries are arrowed above the amino acid sequence.

METHODS. Exon S66 and others that had been trapped in association with it were used to isolate segments of the candidate cDNA by hybridization to normal human fetal brain, placental, monocyte and breast cancer cDNA libraries. Additional fragments were isolated by PCR amplification from known exon sequences to vector ends. In the course of these analyses, other previously trapped exons and cDNAs selected by solution hybridization were incorporated into an extended cDNA sequence. In addition, the exon prediction program Genemark was used to define the location of adjacent candidate transcribed sequences from the genomic sequence. Putative intron–exon boundaries were confirmed by amplification from cDNA and direct sequencing of amplification products. Northern analysis indicates that the transcript from the *BRCA2* gene is large (approximately 10–12 kb), and hence the N terminus of the *BRCA2* protein may well be missing from the above sequence.

To characterize this gene further, exon S66 was used to isolate a series of cDNA clones which represented segments of the *BRCA2* candidate (see Fig. 2 legend). At this stage the initial shotgun sequence data from a 900-kb region thought to contain *BRCA2* was completed at the Sanger Centre and Washington University and became available to us through the public release of the assembled sequence (at ftp: ftp.sanger.ac.uk pub human sequences 13q and ftp: genome.wustl.edu pub gsc1 brca2 from 23 November 1995). From alignment of the cDNA and genomic sequence data, the candidate *BRCA2* gene was found to lie in three sequence contigs which also contained other previously isolated transcribed sequences. The exon and open reading frame prediction program Genemark was used to define putative additional 5′ exons of the gene. Contiguity of the transcription unit was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) on cDNA and sequence analysis. The availability of extensive sequence information at the cDNA and genomic level allowed mutational analysis of further coding regions of the putative *BRCA2* gene in samples from breast cancer families.

A TG deletion and a TT deletion were detected in families CRC B196 and CRC B211 respectively (Table 1). In both families the mutation has been detected by sequencing other individuals with early onset breast cancer who share only the haplotype of 13q microsatellite markers that segregates with the disease. Therefore, the mutations are on the disease-associated chromosomes. A CT deletion was detected in family IARC 3594. This mutation has arisen within a short repetitive sequence (CTCTCT), a feature that is characteristic of deletion/insertion mutations in many genes, and which is presumed to be due to slippage during DNA synthesis. Finally, a T deletion and an AAAC deletion have been found in Montreal 681 and 440, respectively. Both these families include a male breast cancer case, and previous analyses have indicated that the large majority of such families will have *BRCA2* mutations[10]. All these mutations are predicted to generate frameshifts leading to premature termination codons. None of the mutations have been found in over 500 chromosomes from healthy women and are therefore unlikely to be polymorphisms. The identification of several different germline mutations that truncate the encoded protein in breast cancer families that are highly likely to be due to *BRCA2* strongly suggests that we have identified the *BRCA2* gene.

Northern analysis has demonstrated that *BRCA2* is encoded by a transcript of 10–12 kb (data not shown), which is present

in normal breast epithelial cells, placenta and the breast cancer cell line MCF7. This suggests that our present contig of cDNAs covering approximately 7.3 kb (including 300 bp of 3' untranslated sequence) may not include the whole *BRCA2* coding sequence. The known sequence of 2,329 amino acids encoded by the *BRCA2* gene does not show strong homology to sequences in the publicly available DNA or protein databases, and therefore we have no clues to its functions. However, some weak matches were detected including, intriguingly, a very weak similarity to the *BRCA1* protein over a restricted region (amino acids 1394–1474 in *BRCA1*, and 1783–1863 in the portion of *BRCA2* shown in Fig. 2). The significance of this is unclear.

Loss of heterozygosity on chromosome 13q has been observed in sporadic breast and other cancers, suggesting that there is a somatically mutated tumour suppressor gene in this region[11–13]. *BRCA2* is a strong candidate for this gene, and the analysis of a large series of cancers is underway to investigate if *BRCA2* is somatically mutated during oncogenesis.

The identification of *BRCA2* should now allow more comprehensive evaluation of families at high risk of developing breast cancer. However, the roles of environmental, lifestyle or genetic factors in modifying the risks of cancer in gene carriers are unknown, and further studies will be required before routine diagnosis of carrier status can be considered. □

Received 5 December; accepted 7 December 1995.

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ACKNOWLEDGEMENTS. We thank the families for their continuing help and encouragement in this work and the many clinicians who have referred families; F. Dion, R. Carter, K. Anderson, M. Ponder, H. Vasen, J. Feuntein, O. Serova, P. Gwinnam, S. Humphray, M. Leversha, Y. Ramsey, H. King, D. Cain, D. Averill, P. Mitchell, M. Crompton, C. Cochran, J. Marks, D. Iglehart, R. Wiseman, J. Lancaster and the members of the Human Sequencing teams at the Sanger Centre and the Genome Sequencing Centre at Washington University for making their data publicly available; M. Schutte and S. Kern for discussions; and C. Marshall, C. Cooper and P. Garland for encouragement. This work was supported by the Cancer Research Campaign, BREAKTHROUGH Breast Cancer Charity and the Jean Rook Appeal, the Institute of Cancer Research, the Wellcome Trust, the Medical Research Council, the Imperial Cancer Research Fund, the U.S. Army, Duke University SPORE in Breast Cancer, the National Cancer Institute, the Icelandic Cancer Research Fund, the Nordic Cancer Union, the Ligue Nationale contre le Cancer, the Dutch Cancer Society, the Cancer Research Society, the Canadian Breast Cancer Foundation, the Fonds de la recherche en Sante du Quebec, and the Canadian Genetic Diseases Network. Access to BRCA2 sequences can be obtained from http://www.ncbi.nlm.nih.gov

RETRACTION

Cloning and functional expression of a rat heart K_{ATP} channel

M. L. J. Ashford, C. T. Bond, T. A. Blair & J. P. Adelman

Nature **370,** 456–459 (1994)

In this letter we described the cloning and expression of an inward rectifier potassium-channel subunit from rat heart (Kir 3.4) which, when transfected into HEK293 and BHK21 cells, endowed them with ATP-sensitive potassium channels. Since this paper appeared, we have not been able regularly to reproduce those findings. In addition, the data presented by Krapivinsky *et al.*[1] presents a compelling argument that Kir 3.4 is an intrinsic component of the channel underlying I_{KACh} in atrium, and that it does not contribute to the channel underlying cardiac I_{KATP}. Therefore, we cannot support our previous statement that Kir 3.4 represents a subunit of cardiac K_{ATP} channels. □

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Seminars in Oncology

Vol 23, No 1, Suppl 2 February 1996

The Genetics of Familial Breast Cancer

L.A. Cannon-Albright and M.H. Skolnick

Because studies of breast cancer patients and their relatives provide statistical evidence for involvement of autosomal dominant genes, the identification of specific genetic effects has long been the focus of efforts to identify women at exceedingly high risk. BRCA1, a gene that confers greatly increased susceptibility to breast and ovarian cancer, was isolated in 1994, capping an intense analysis by a large number of groups of a complex phenotype. BRCA1 is a large gene and shows only limited homology to other known genes. Near the amino terminus of the predicted protein is a RING finger motif. In addition, a leucine heptad repeat appears in the interior of the sequence. Several groups have looked extensively for somatic BRCA1 mutations in breast and ovarian tumors. The frequency of somatic mutations in ovarian tumors is low, and to date no somatic mutations have been found in breast tumors. More research is needed to define the role of BRCA1 in sporadic tumors. A second locus associated with predisposition to early onset breast cancer, BRCA2, has been localized to chromosome 13q. Positional cloning of this gene is well advanced and analysis of its biology and mutation spectrum is eagerly awaited. As the BRCA1 and BRCA2 genes are characterized further, a diagnostic test for breast cancer susceptibility becomes feasible.

BREAST CANCER is the most common cancer among American women. Because metastatic breast cancer is an incurable disease, efforts to decrease breast cancer mortality have focused on early diagnosis and improved treatment. Identification and analysis of a susceptibility locus allow detection of susceptible individuals before a tumor develops as well as greatly increase our understanding of the initial step that eventually leads to cancer. As susceptibility loci are often altered during tumor progression, identification and cloning of such loci could be of importance in developing cancer therapies.

Breast cancer has long been recognized to be, in part, a familial disease, but the nature of the genetic susceptibility that predisposes women is largely unknown.[1] Studies have shown that a woman's risk of developing breast cancer is increased if one or more first-degree relatives have had breast cancer. The increased risks are greatest if affected relatives have had premenopausal or bilateral breast cancer.[2-6] Numerous investigators have examined the inheritance of breast cancer and concluded that the data are most consistent with dominant inheritance for a major susceptibility locus or loci.[7-12]

Genetic factors contribute to some proportion of breast cancer incidence, estimated to be 5% to 10% of all cases, and up to 25% of all cases diagnosed before the age of 30 years.[13] Breast cancer has been subdivided into early onset and late onset based on the inflection in the age-specific incidence curve at about the age of 50 years. In 1990, a breast cancer susceptibility gene, BRCA1, was localized to chromosome arm 17q.[14] An intense effort to isolate BRCA1 culminated in the report of Miki et al in 1994.[15] A second locus, BRCA2, was mapped to chromosome arm 13q at approximately the same time.[16] BRCA2 appears to account for a proportion of early onset breast cancer, which is roughly equal to that resulting from BRCA1. BRCA2, however, appears to have much less of an influence on ovarian cancer risk than BRCA1. The remaining susceptibility to early onset breast cancer is likely attributable to unmapped genes for familial cancer and rare germline mutations in genes such as TP53, which encodes the tumor suppressor protein p53.[17] It also has been suggested that heterozygote carriers of defective forms of the gene predisposing to ataxia telangiectasia are at higher risk for

From the Division of Hematology/Oncology, Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, UT; and Myriad Genetics, Salt Lake City, UT.

Supported by Department of Defense Grant No. DAMD 17-94-J-4260 and by National Institutes of Health Grants No. RR-00064, CA-55914, CA-65673, CA-64477, and CA-48711.

Address reprint requests to L.A. Cannon-Albright, PhD, Genetic Epidemiology, Suite D2, 391 Chipeta Way, Salt Lake City, UT 84108.

0093-7754/96/2301-0208$05.00/0

breast cancer.[18,19] The recent isolation of this gene will allow testing of this hypothesis.[22] Late onset breast cancer is often familial in origin, but the percentage of such cases that are due to genetic susceptibility is unknown. This population remains understudied, and mutation screening for both BRCA1 and BRCA2 will clarify the role of inherited susceptibilities in these families.

RISKS FOR BRCA1 MUTATION CARRIERS

The early detection of women at high risk provides a promising approach for reducing the high incidence and mortality associated with breast and ovarian cancer. These women, once identified, can be targeted for more aggressive prevention programs. The isolation of the BRCA1 gene now allows us to proceed with this identification and understanding. The lifetime risk of cancer in BRCA1 mutations carriers is high. Anecdotal evidence suggests that prostate cancer may be associated with BRCA1.[21] Ford et al[22] estimated the risks of breast and ovarian cancer from the occurrence of second cancers in individuals with breast cancer in BRCA1 families and examined the risks of other cancers in carriers. The estimated cumulative risk of breast cancer in gene carriers was 87% by the age of 70 years. The estimated cumulative risk of ovarian cancer was 44% by the age of 70 years. Significant excesses of risk were also observed for colon cancer and prostate cancer, and not for cancers of other sites.

Using the model of Easton et al[23] for penetrance of BRCA1 and the estimates of heterogeneity reported by Easton et al,[24] it is possible to estimate prior probabilities of a particular constellation of cases being due to the BRCA1 gene. These are shown in Table 1. It is clear from these estimates that a single affected breast cancer case has a very low prior probability of having a mutation, sister pairs involving at least one ovarian cancer have close to 50% prior probability, and clusters of breast and ovarian cancer in a family indicate a greater than 80% prior probability of a BRCA1 mutation.

Heterogeneity of ovarian cancer risk has been described by Easton et al[24] in a study using family data from the International Breast Cancer Linkage Consortium. The overall risk of ovarian cancer in BRCA1 carriers was estimated to be 44% by the age of 70 years. However, there was statistically significant evidence for heterogeneity of risk,

Table 1. Estimated Prior Probabilities of a Particular Constellation of Cases Being Due to the BRCA1 Gene[25]

Criterion, Cancer Diagnoses, Age	Prior Probability Case Has a BRCA1 Mutation
Single affected	
Breast, <30 yr	0.12
Breast, <40 yr	0.06
Breast, 40–49 yr	0.03
Ovarian, <50 yr	0.07
Sister pairs	
Breast < 40 yr	0.37
Breast 40–49 yr	0.20
Breast < 50 yr	0.46
Ovarian < 50 yr	0.61
Families	
Breast only, >2 cases diagnosed at <50 yr	0.40
>1 breast with >0 ovarian	0.82
>1 breast with >1 ovarian	0.91

which suggested that in 89% of families carriers had an elevated, modest risk for ovarian cancer of 23%, and that in the remaining minority of families the carriers had a lifetime risk of ovarian cancer of 85%. To identify a genetic basis for this observed heterogeneity of risk, the distribution of mutations in families with heterogeneous ovarian cancer risk has been examined. The original report[25] suggested that although mutations were found in samples from each of three groups of families (defined by the proportion of affected women who had ovarian cancer), mutations in the 3′ portion of the gene were associated with families with a lower proportion of ovarian cancers in affected women ($P = .08$). More recent analysis of 75 families further supports this significant association of location ($P < .01$) (D.E. Goldgar, personal communication, September 1995). In a similar study of the six most common recurrent mutations, 61 geographically diverse families were compared. There was no significant mutation-specific association with age at onset of disease; however, mutation-specific differences in relative proportions of cases of breast and ovarian cancer ($P = .06$) were again noted (S.L. Neuhausen and D.E. Goldgar, personal communication, October 1995).

MUTATION SCREENING FOR BRCA1

The findings of a large collaborative group of investigators in identifying mutations in the

BRCA1 gene were published in 1995 and gave an initial view of the mutation profile of BRCA1.[25] The screening has continued, and more up-to-date results are presented here. In addition, studies of specific mutations provide definition of mutation-specific differences in penetrance.

BRCA1 is a large gene, containing 5,592 nucleotides spread over approximately 100,000 bases of genomic DNA. It is composed of 22 coding exons and produces a protein of 1,863 amino acids. Much of BRCA1 shows no homology to other known genes, with the exception of a 126 nucleotide sequence at the amino terminus, which encodes a RING finger motif, a configuration characteristic of the RING-1 gene.[26] This motif is found in other proteins that interact with nucleic acid and/or form protein complexes, which may suggest a role for BRCA1 in DNA transcription. In addition, a leucine heptad repeat appears in the interior of the sequence.

Mutations in BRCA1 result from several different DNA alterations. Two classes of mutations result from substitution of a single nucleotide. If the substitution changes a single amino acid but does not affect the remainder of the protein translation, it is termed a *missense* mutation. *Nonsense* mutations occur when the nucleotide substitution produces a stop codon (TGA, TAA, or TAG), and translation of the protein is terminated at that point. Mutations in which one or more nucleotides are either inserted or deleted are termed *frameshift* mutations. If the number of bases inserted or deleted is not divisible by three, a change in the reading frame alters the remainder of the translation of the protein being altered; most often a stop signal is encountered prematurely. Another class of mutation, *intron/exon splice-site* mutations, can result from either single-base changes or the insertion or deletion of one or more nucleotides in the intronic sequence. Splice-site mutations cause abnormal inclusion or exclusion of DNA in the coding sequence, resulting in an aberrant protein. A final class of mutation occurs when a mutation in a gene's regulatory region causes reduction or loss of protein synthesis from the mutant chromosome. Such regulatory mutations usually occur outside of the coding sequence of a gene and can be either nucleotide substitutions or insertion/deletion events.

A combination of different methods can be used to detect mutations, including direct DNA sequencing (either manual sequencing or automated fluorescent sequencing), single-strand conformation polymorphism assay or other approaches based on detection of mismatches between the two complementary DNA (cDNA) strands, including clamped denaturing gel electrophoresis and heteroduplex analysis. A review of currently available methods of detecting DNA sequence variation can be found in Grompe.[27]

Table 2. One Hundred BRCA1 Mutations Identified by Screening in 195 Samples

Mutation	Frequency	Cumulative %
185 del AG	21	11
5,382 ins C	20	21
4,184 del 4	8	25
T→G intron 5 splice	7	29
1,294 del 40	6	32
Cys 61 Gly	6	35
3,166 ins 5	4	37
3,449 del 4	4	39
3,598 del 11	4	41
Arg 1,443 Ter	4	43
Met 1,652 Ile	4	45
1,499 ins A	3	47
3,875 del 4	3	48
14 mutations	2	63
73 mutations	1	100

In a large collaborative effort, 100 unique mutations have been identified to date in 195 individuals. Table 2 shows these mutations and the observed frequencies. The majority of mutations identified are frameshifts that account for 51% of the 100 different mutations found to date in BRCA1 and in 62% of all samples shown to have BRCA1 mutations. Of the total of 100 independent individuals with mutations, 84% are frameshift, nonsense, splice, or regulatory mutations, which result in a truncated protein product. The high frequency of protein terminating mutations and the many recurrent mutations found suggest the possibility of a relatively simple diagnostic test for BRCA1 mutations. More data are being accumulated to allow estimation of the sensitivity and specificity of such a diagnostic testing procedure and to estimate age-specific risks for breast cancer, ovarian cancer, and cancers of other sites.

BRCA1 MUTATIONS IN TUMORS

Germline mutations of the BRCA1 tumor suppressor gene on chromosome 17q are involved in a

significant fraction of hereditary breast and ovarian cancers. Allelic deletions that include the BRCA1 locus are common in breast and ovarian cancers, implying that somatic mutations of this gene might play an important role in the more common sporadic forms of these tumors as well. Up to 80% of sporadic breast cancers and a similar number of sporadic ovarian cancers contain allelic deletions on 17q.[28-32] Concurrent with the presentation of the isolation of BRCA1, Futreal et al[33] found that breast and ovarian cancers selected for 17q LOH did not appear to contain somatic mutations in BRCA1. These investigators reported four mutations in their collection of tumors, but all were present in the germlines of individuals. Among many similar studies that found no mutations, there has been little evidence for somatic mutations in sporadic ovarian cancers, and a somatic mutation in a sporadic breast cancer has yet to be reported. Merajver et al[34] reported finding somatic mutations in the DNA of four of 47 tumors that also had LOH at a BRCA1 intragenic marker. Hosking et al[35] concurrently reported a somatic mutation in the DNA of one of 17 sporadic ovarian tumors previously demonstrating LOH on 17q.

MALE BREAST CANCER

Breast cancer is rare in men and family history of the disease is a risk factor for men. Carcinoma of the breasts is approximately 100-fold less common in men than in women and accounts for approximately 1% of all male cancers. BRCA2 is thought to account for some families with increased risk of breast cancer, especially those which include male breast cancer. The risk of female breast cancer in relatives of men with the disease may be increased. Male relatives of female patients are at increased risk.[36,37] Mutations in the androgen receptor gene have been found in at least two families with familial male breast cancer.[38,39] Male breast cancer has been shown to be rare in families with BRCA1 mutations.[40]

BRCA2

BRCA2 was the second breast cancer susceptibility gene to be localized. It was localized to a region of chromosome arm 13q.[16] Families that carry a mutated BRCA2 gene have an increased risk of breast cancer and a slightly increased risk of ovarian cancer. BRCA2 families often have cases of male breast cancer. Studies similar to those presented for BRCA1 will not be possible until BRCA2 is isolated and mutation screening can be performed. Initial analysis suggests that BRCA2 may increase risk for cancer of other sites. A study that used the two most extensive BRCA2 kindreds known to date suggests elevated risks for ovarian cancer (RR 17.7), cancer of the larynx (RR 7.7), and prostate cancer (2.9) (D.E. Goldgar and D.F. Easton, personal communication). Risks for breast cancer appear to be similar to those for BRCA1. Excesses of stomach and thyroid cancers also have been observed, although the significance is not yet understood.[41,42]

CONCLUSION

The identification of inherited susceptibilities for breast cancer opens new possibilities for detection, screening, and treatment. The genes will greatly enhance our understanding of breast and ovarian cancer, the interaction of genes and environment, and tumorigenesis.

NOTE ADDED IN PROOF

The BRCA2 gene was isolated in 1995.[43,44]

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GENOMICS 36, 86–99 (1996)
ARTICLE NO. 0428

Generation of an Integrated Transcription Map of the *BRCA2* Region on Chromosome 13q12–q13

FERGUS J. COUCH,* JOHANNA M. ROMMENS,†,‡ SUSAN L. NEUHAUSEN,§ CAROLE BÉLANGER,¶ MARTINE DUMONT,¶ KENNETH ABEL,‖ RUSSELL BELL,** SIMON BERRY,** ROBERT BOGDEN,** LISA CANNON-ALBRIGHT,§ LINDA FARID,* CHERYL FRYE,** THOMAS HATTIER,** TERESA JANECKI,** PING JIANG,** ROBERT KEHRER,** JEAN-FRANCOIS LEBLANC,¶ JODI MCARTHUR-MORRISON,† DAVID MCSWEENEY,† YOSHIO MIKI,†† YI PENG,* CAROLLE SAMSON,¶ MARIANNE SCHROEDER,** SARAH C. SNYDER,** MICHAEL STRINGFELLOW,** CARRIE STROUP,** BRAD SWEDLUND,** JEFF SWENSEN,§ DAVID TENG,** SANJAY THAKUR,* THANH TRAN,** MARTINE TRANCHANT,¶ JANE WELVER-FELDHAUS,** ALEXANDER K. C. WONG,** HIROAKE SHIZUYA,‡‡ FERNAND LABRIE,¶ MARK H. SKOLNICK,§,** DAVID E. GOLDGAR,§,[1] ALEXANDER KAMB,** BARBARA L. WEBER,* SEAN V. TAVTIGIAN,**,[2] AND JACQUES SIMARD[¶,2,3]

*Department of Medicine, Department of Pathology, and Department of Genetics, University of Pennsylvania, Philadelphia, Pennsylvania 19104; †Department of Genetics, Research Institute, The Hospital for Sick Children, and ‡Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, M5G 1X8, Canada; §Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah 84132; ¶Laboratory of Molecular Endocrinology, CHUL Research Center and Laval University, Quebec City, Quebec, G1V 4G2, Canada; ‖Department of Human Genetics, University of Michigan, Ann Arbor, Michigan 48109; **Myriad Genetics Inc., 390 Wakara Way, Salt Lake City, Utah 84108; ††Division of Biochemistry, Cancer Institute, Tokyo, Japan; and ‡‡Division of Biology, California Institute of Technology, Pasadena, California 91125

Received March 22, 1996; accepted May 29, 1996

An integrated approach involving physical mapping, identification of transcribed sequences, and computational analysis of genomic sequence was used to generate a detailed transcription map of the 1.0-Mb region containing the breast cancer susceptibility locus *BRCA2* on chromosome 13q12–q13. This region is included in the genetic interval bounded by *D13S1444* and *D13S310*. Retrieved sequences from exon amplification or hybrid selection procedures were grouped into physical intervals and subsequently grouped into transcription units by clone overlap. Overlap was established by direct hybridization, cDNA library screening, PCR cDNA linking (island hopping), and/or sequence alignment. Extensive genomic sequencing was performed in an effort to understand transcription unit organization. In total, approximately 500 kb of genomic sequence was completed. The transcription units were further characterized by hybridization to RNA from a series of human tissues. Evidence for seven genes, two putative pseudogenes, and nine additional putative transcription units was obtained. One of the transcription units was recently identified as BRCA2 but all others are novel genes of unknown function as only limited alignment to sequences in public databases was observed. One large gene with a transcript size of 10.7 kb showed significant similarity to a gene predicted by the *Caenorhabditis elegans* genome and the *Saccharomyces cerevisiae* genome sequencing efforts, while another contained a motif sequence similar to the human 2′, 3′ cyclic nucleotide 3′ phosphodiesterase gene. Several retrieved transcribed sequences were not aligned into transcription units because no corresponding cDNAs were obtained when screening libraries or because of a lack of definitive evidence for splicing signals or putative coding sequence based on computational analysis. However, the presence of additional genes in the BRCA2 interval is suggested as groups of putative exons and hybrid selected clones that were transcribed in consistent orientations could be localized to common physical intervals.

INTRODUCTION

Transcript maps of chromosomal regions are important milestones to understanding the human genome (Collins and Galas, 1993). Physical and genetic maps provide the framework for detailed study of genomic regions while transcript maps are essential for the characterization of all genes present and an understanding of their genomic organization. Transcript

[1] Current address: Unit of Genetic Epidemiology, International Agency for Research on Cancer, Lyon Cedex 08, France.
[2] These authors should be considered equal contributors.
[3] To whom correspondence should be addressed. Telephone: (418) 654-2264. Fax: (418) 654-2735. E-mail: Jacques.Simard@crchul.ulaval.ca.

0888-7543/96 $18.00

maps also play a role in the identification of candidate genes for mapped disease genes.

Genes or gene fragments can be identified from genomic DNA by a variety of methods. The most common approaches are (1) exon amplification (Buckler *et al.*, 1991), (2) hybrid selection (Parimoo *et al.*, 1991; Lovett *et al.*, 1991), (3) analysis of genome sequence (Uner-Bacher and Mural, 1991), and (4) direct cDNA library screening (Brody *et al.*, 1995). The advantages and disadvantages of these methods have been described (Hochgeschwender, 1992; Harshman *et al.*, 1995). As it is unlikely that a single method will identify all the transcripts within a given genomic interval (Harshman *et al.*, 1995), we sought to develop a detailed transcription map of the BRCA2 region by employing the first three of these techniques.

In late 1994, the breast cancer susceptibility locus *BRCA2* was mapped to the 6-cM region between the genetic loci *D13S289* and *D13S267* (Wooster *et al.*, 1994). This interval was subsequently reduced to 4 cM by identification of recombination events in linked families with early onset breast cancer using the markers D13S289 and D13S260 (Thorlacius *et al.*, 1995). Subsequent physical mapping revealed this region to be approximately 2 Mb in size, a region small enough to attempt a positional cloning strategy. A physical map of the 1.5-Mb region between *D13S1444* and *D13S310* was constructed (see below). Coincident with ongoing genetic and physical mapping studies, analysis of sporadic pancreatic cancer tumors resulted in identification of a single homozygous deletion of 250–300 kb located within the BRCA2 interval between *D13S260* and *D13S171* (Schutte *et al.*, 1995a,b). As deletions at this interval are extremely rare (Teng *et al.*, 1996), the presence of the homozygous deletion within the delineated region defined by genetics suggested the existence of a tumor suppressor gene, possibly BRCA2. Also during the course of our study 900 kb of genomic sequence from the BRCA2 region was made publicly available by the Sanger and Washington University sequencing centers. Most recently, a portion of the BRCA2 cDNA sequence (7.3 kb of an estimated 10–12 kb) was reported (Wooster *et al.*, 1995). In parallel, the complete coding sequence and the exonic structure of the BRCA2 gene, which is composed of 27 exons distributed over approximately 70 kb of genomic DNA, have been determined and deposited in GenBank (Accession No. U43746) (Tavtigian *et al.*, 1996).

We present a transcription map of the BRCA2 region integrated with the physical and genetic maps of the region. The physical map spans the 1.5-Mb interval between *D13S1444* and *D13S310*. The most thoroughly analyzed segment of the transcription map spans the 750-kb interval bounded by *D13S1699* and *D13S171*. In addition to the BRCA2 gene, we have identified 17 transcription units. Furthermore, several expressed segments including unique exons and cDNA fragments not contained within the assigned transcription units have been isolated and accurately mapped to the region.

MATERIALS AND METHODS

Physical map assembly. Yeast artificial chromosome (YAC) clones from the Centre d'Etude du Polymorphisme Humain (CEPH) containing short tandem repeat (STR) markers D13S289, D13S290, D13S171, D13S260, and D13S267 were identified from compiled maps at GenBank. The YACs were obtained from Research Genetics Inc. (Huntsville, AL), propagated, and verified using the STRs initially determined to be linked to BRCA2 region. Additional STRs were obtained from CEPH, and sequence-tagged sites (STSs) were generated from YAC ends either by YAC vector-*Alu* PCR using primers as described (Neuhausen *et al.*, 1994) or by YAC vector-random primer PCR (Swensen 1996). These STSs were used to identify overlaps among the YACs. YAC end STSs were checked on the somatic cell hybrids NA11689 and NA11575 (Coriell Institute Camden, NJ) to ensure that all of the YAC ends were on 13q.

An initial, incomplete set of P1s and BACs in the region was identified using STRs and YAC-derived STSs to screen the Genome Systems P1 library and Simon Laboratory BAC library "A" (Shizuya *et al.*, 1992). P1 and BAC clone DNAs were prepared and ends were sequenced as described (Neuhausen *et al.*, 1994). Clone overlaps and relative orientations were determined by STS content mapping. Nonrepetitive markers from the edges of the developing clone contigs were used to screen the P1 and BAC libraries again. Once the PAC library became available from Genome Systems, PAC screening was substituted for P1 screening. In this way, the developing P1/BAC/PAC clone contigs were expanded until a single contig spanning the entire genetically defined interval was completed. Di-, tri-, and tetranucleotide repeat markers were cloned from the P1s, BACs, and PACs by hybrid capture using biotinylated repeat-containing oligos (Swensen 1996). STR primer sequences and sizes of the products are given in Table 2. They are also available from the Genome Data Base (GDB).

Restriction analysis of P1s, PACs, and BACs. Five hundred nanograms of DNA from each clone was digested with *Eco*RI or *Hin*dIII and fractionated in 0.5% agarose gels. The restriction fragments were visualized with ethidium bromide staining under ultraviolet light, and clone lengths were determined by adding the sizes of individuals restriction fragments, excluding those corresponding to vector fragments. The extent of overlap among neighboring P1s, BACs, and PACs was calculated by adding the sizes of shared restriction fragments.

Identification of STRs. STRs were identified by three methods: (1) probing immobilized cosmid DNA with a (CA)n oligomer to identify repeats; (2) direct sequencing of P1 clones with a (CA)n oligomer; and (3) hybrid capture of P1, BAC, and PAC fragments containing di-, tri-, and tetranucleotide repeats. Followed cloning and sequencing, oligonucleotides were selected and used in PCR as described (Neuhausen *et al.*, 1994). Details of each identified STR are indicated in Table 2.

Hybrid selection. Randomly primed cDNA was prepared from poly(A)$^+$ RNA of mammary gland, ovary, testis, fetal brain, and placenta tissues and from total RNA of the Caco-2 cell line (ATCC HTB 37). Hybrid selection with pooled cDNA was carried out for two consecutive rounds of hybridization to immobilized P1 or BAC DNA as described previously (Tavtigian *et al.*, 1996; Rommens *et al.*, 1995). Two to four overlapping P1 and/or BAC clones were used in individual selection experiments. Approximately 200 to 300 individual colonies from each ligation (from each 250 kb of genomic DNA) were picked and gridded into microtiter plates for ordering and storage. Cultures were replica transferred onto Hybond-N membranes (Amersham). Initial analysis of the cDNA clones involved a prescreen for ribosomal sequences and subsequent cross screenings for detection of overlap and redundancy.

Plasmids from 25 to 50 clones from each selection experiment that did not hybridize in the prescreening phase were isolated for further

analysis. The retrieved cDNA fragments were verified to originate from individual starting genomic clones by hybridization to restriction digests of DNAs of the starting clones. The clones were tentatively assigned to groups based on the overlapping or nonoverlapping intervals of the genomic clones. The fragments were also hybridized to genomic DNAs from human leukocytes and from the rodent hybrid cell GM10898A (NIGMS) that contains human chromosome 13.

A secondary round of hybrid selection was also performed. Biotinylated DNA from 10 genomic clones, positioned between P1-759-D10 and BAC B213E7 on the physical map, was hybridized to pooled cDNA from human mammary gland, brain, lymphocyte, and stomach in individual experiments as previously described (Tavtigian *et al.*, 1996). Two X 96-clones were isolated from each experiment. Each of the 1920 clones was sequenced, checked for repetitive sequence, and assessed for redundancy by comparison to other DNA databases and cDNA sequence from the BRCA2 region. Contigs of clones were assembled where overlapping sequence was identified. Many of the hybrid selected clones identified in Fig. 1 represent hybrid clone contigs rather than individual clones.

Exon amplification. Exon amplification was performed according to the methodology described (Church *et al.*, 1994). A minimal overlapping set of BACs, PACs, and P1s from the BRCA2 region was utilized. Three different experiments were undertaken: (1) a single pool of 10 genomic clones; (2) six pools of 3 overlapping genomic clones each; and (3) 10 individual genomic clones. Genomic clones were digested with *Pst*I or *Bam*HI and *Bgl*II and ligated into *Pst*I or *Bam*HI sites of the pSPL3 splicing vector. End products of exon amplification were cloned into the pAMP1 plasmid with the Uracil DNA Glycosylase cloning system (Life Technologies, Inc.). Six thousand clones were picked, propagated in 96-well plates, stamped onto filters, and analyzed for the presence of vector and repeat sequences by hybridization. Each clone insert was amplified by PCR and tested for redundancy and human clone specificity by hybridization to membranes with exon DNA and dot blots to the parent genomic DNA clone. Candidate exons were sequenced, compared to DNA sequence databases, and used as probes for screening cDNA libraries.

cDNA library screening. To provide a preliminary screen of cDNA libraries for hybrid selected clones, PCR was performed using specific oligonucleotides derived from the sequences of each of the retrieved cDNA clones using DNA from five λgt11 cDNA libraries (human mammary gland (HL 1037b), human breast (HL 1061b), ZR-75-1 human breast cancer cells (HL 1059b), human ovary (HL 1098b), and human testis (HL1010b) (Clontech)) as template. Hybrid-selected clones present in cDNA libraries (as detected by this approach) were used as probes to screen the corresponding positive libraries. Hybrid clones for which PCR was not attempted were used as probes to screen a pool of the libraries described above (2×10^5 clones for each library) as previously described (Rommens *et al.*, 1995). Prehybridization and hybridization were performed at 42°C in 50% formamide, 5× SSPE, 0.1% SDS, 5× Denhardt's mixture, 0.2 mg/ml denatured salmon testis DNA, and 2 μg/ml poly(A). Dextran sulfate (4%, v/v) was included in the hybridization solution only. The filters were rinsed in 2× SSC for 10 min at room temperature and then washed in 2× SSC/0.1% SDS for 30 min at 60°C followed by two washes in 1× SSC/0.1% SDS for 20 min each at 60°C. The positive phages were retested for second and third screenings, as required, to obtain purified plaques for subcloning or sequencing.

Unique exons were amplified by PCR, double labeled with [α-^{32}P]-dCTP and [α-^{32}P]dGTP, and screened against a pool of libraries (HepG2 liver, breast, mammary, placental, and testis (Clontech)) containing 200,000 clones from each. The HepG2 and testis libraries, an ovarian oligo(dT) primed library (Stratagene), and a random-primed breast library (Clontech) were also screened individually if the pooled screen was negative. Prehybridization and hybridization were performed in 10 m*M* NaCl, 5% SDS, 10% dextran sulfate, 100 μg/ml of salmon sperm DNA at 65°C. Membranes were washed in 3× SSC, 0.5% SDS at 65°C for a total of 60 min and exposed to film at −70°C overnight. The positive phages were retested for second and third screenings to obtain purified plaques for PCR-based sequencing.

DNA sequencing. Expressed sequences retrieved during the first round of hybrid selection and many of the clones retrieved from cDNA libraries were sequenced by with the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7 Sequencing Kit (Pharmacia Biotech Inc.). Alternatively, expressed sequences retrieved during the second round of hybrid selection, many of the clones retrieved from cDNA libraries, and all genomic DNA subclones obtained from the P1s, BACs, and PACs were sequenced on ABI 377 sequencers using ABI Prism dye terminator cycle sequencing kits (Perkin–Elmer). Finally, sequencing directly from P1, BAC, or PAC templates utilized either the Cyclist DNA sequence kit (Stratagene) or the AmplicCycle DNA sequencing kit (Perkin–Elmer) interchangeably.

Sequence analysis. All of the sequence data generated over the course of this project were assembled into a Genetic Data Environment (GDE) (Smith *et al.*, 1994) database or Wisconsin Sequence Analysis Package GCG program (Version 8, September 1994, Genetics Computer Group, 575 Science Drive Madison, WI 53711). Sequence alignment, assembly, and the parsing of exons across genomic sequences were performed within GDE. BLAST (Altschul *et al.*, 1990) and FastA (Pearson and Lipman, 1988) searches against both local and remote databases were also initiated from within GDE. Sequences from cDNAs described in this work have been submitted to the NCBI databases under the GenBank Accession numbers listed in Table 3.

Northern analysis. RNA from HepG2 (ATCC HB8065), T-47D (ATCC HTB 133), MCF-7 (ATCC HTB 22), JEG-3 (ATCC HTB 36), Caco-2 (ATCC HTB 37), A293 (ATCC CRL 1573), and HeLa (ATCC CCL2) cell lines was isolated using the TRI reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA was electrophoresed on a 1.2% agarose–formaldehyde gel and transferred to a GeneScreen Plus membrane (NEN, Dupont). Following UV cross-linking, the membranes were hybridized with probes labeled by random priming with the same hybridizing solutions described for the cDNA library screening, except for the presence of 1% SDS instead of 0.1% SDS. The membranes were washed twice in 2× SSC/0.1% SDS at 20°C for 30 min followed by a stringency wash in 0.1× SSC/0.1% SDS at 50°C for 30 min. RNA hybridization was subsequently performed as described above with MTN filters from Clontech. RNA probes were removed between hybridizations by heating membranes to 95°C in 0.02× SSC and 0.01% SDS.

RESULTS AND DISCUSSION

Physical and Genetic Map Integration

Two complete genomic clone contigs spanning the BRCA2 region as defined by genetic loci were assembled. A yeast artificial chromosome contig was obtained using the "Infoclone" database of the GDB. The relative positions and overlap of these YACs were verified and refined by STS content mapping using STR markers and YAC end-derived STSs. Cosmid libraries constructed from YACs 964B2, 931F4, 979H8, 951A3, 746G10, and 941D4 were screened with a CA_n probe to identify new STRs. Cosmid ends were also sequenced to provide additional STSs for P1, BAC, and PAC library screening. An incomplete set of P1 and BAC clones of the region was obtained using the STRs and YAC-derived STSs by screening the Genome Systems P1 library and the Simon Laboratory BAC library "A" (Shizuya *et al.*, 1992). Initial groups of P1 and BAC clones were subsequently connected by using STSs developed from their respective ends. Once the PAC library became available from Genome Systems, PAC screening was substituted for P1 screening. Table 1 contains primer sequences and product sizes for the STSs used

TABLE 1

Primer Sequences and Sizes for the Sequence-Tagged Sites Derived from P1, BAC, and PAC Clones in the BRCA2 Region

STS name	Forward primer	Reverse primer	STS size (bp)
P1-1101-F9 SP6	GGG TAA CTG GAC GTA AAG AC	GTG AGT TGA AAT GCT GTC TG	186
P1-1101-F9 T7	GAG TTC ACG ATT AAT TCT TAG G	GTG GTT CTA CCA AAC AGA CA	163
P1-981-C5 SP6[a]	Repetitive	Repetitive	
P1-981-C5 T7	CTA TTC CCA TGA AAT AAA GG	GTT GCT TCA GAG GTT AAG TC	178
B52F10 SP6	GCC TAA AAG TTG TAG TGC TG	AAT GAG GGG AAT CGA GTC TA	144
B52F10 T7	ATT AGG AGG GAA AAT AAG AAG G	CAA GCC TGG ACA TCA ATG AG	126
P1-759-D10 SP6	CTT TAC CGT GGA AAC GCT TA	TGA GAG GTG AGG ATG TCT GC	181
P1-759-D10 T7	GCT ACA GCC ACA AAC TTA TGA	GAG TAC TGG GCA CAG AAA GA	249
P1-931-D1 SP6	AGA CAG AGA ATC TCA ACT GG	TTT GAT TTT CAC AGC AGA TG	155
P1-931-D1 T7[a]	Repetitive	Repetitive	
P1-106-A2 SP6	GTG ACA GTA AGC TTC CTT G	AAA GAA CAT CCT TAG TTT GAC	153
P1-106-A2 T7	CAG GAA GAA CTG GAG GTT AG	ACG CTG CTT TGT TAT TTA GG	200
P1-1282-C12 SP6	CCA GGC TGA GCA ACA GAG AA	TGA CAG AGC AAG ACC CCA TC	150
P1-1282-C12 T7	ACT GCT GAT GAG TTT TGG TG	ACA GTG GTA GAC CAT CCA TA	200
B489G4 SP6	GAA TGA GGG CAA GGA ACA C	TTG ACA GCA AGC CAG TGA TA	227
B489G4 T7	GCT TTG AAT GTG GCC CAA CA	ATA TGT GTA AGA CGC GGG TG	177
PAC-25-7K SP6	TAT TAT CTC TTT GAA GTG G	ACT ACT AAA TTC CTG CTA C	172
PAC-25-7K T7	TTG TTG TTT TGT CTG AGG	TAC CCA GCT ACT TGA AGA	141
P1-294-F6 SP6[a]	Repetitive	Repetitive	
P1-294-F6 T7	GCT TAC AAT ACG CAA CTT AC	AAT ATC TTA AAT GGT CAC AGG	196
P1-919-D6 SP6	AGA TGC TTA CTG GCA CTT AC	CAA TAT AGG AGG CCT AGT GTC	151
P1-919-D6 T7	TGA GAC CTT TTA TCA TCT GC	CAA GCT AAT CTG ACC AAG TG	247
B86E4 SP6	GGTATTACACCTGTTTTGCTC	GATGGATAATTCATCCCATAAC	130
B86E4 T7	CCC TCC TCC TAA GGT CAC TA	CCG TCT CAC TGG AGA GAT AA	118
B213E7 SP6	ACC AAC AAT CTC TTC AAG G	ACC AAC ACT GCT GAC ACC	240
B213E7 T7	GCT CCC TCT TAT TCC TTG	CTG TAT TTC AGG CAC TGG	220
PAC-38-14A SP6	AAT GGA GAG GAG GCT GTT A	GAA AAT TAA GAG CCC AGA A	75
PAC-38-14A T7	ATA CAT ATT TTC TCA AGG GAT A	CAT TGG CTT TTT GTC CTCT	98
P1-1149-C3 SP6	GGG TAG AAC AGG CAT TCG TC	CCT CAA CTT AGA TGG TGC CAG	100
YAC-979H8R[b]	CAG GAG GCT CAC AGC TCA GG	CAA GCA GAG CCA AAG GTG AG	120
P1-546-D4 SP6	CAA TAA AGG AAT ATG TGT AGA TAC	TCC ATC AAC TTA CTA TAC AAC TCC	150
P1-546-D4 T7	CCA CCC CTG CAT GGA CTC TG	CAT GGG TGT CAC GTG GAC TC	130
B2767G6 SP6	TAT TTC ATC CAA CCA TGT GC	GGA AAC CCA TTC TAT TAC AG	174
B276G6 T7	CAA GAC AGT GCA AGT GGT AG	TCC TCA TCG GAG TCG TCA	201
B484C6 SP6	AAC TGA GGT CTG CTA TTC A	ATT CTC TTC TGA CTT GGT A	179
B484C6 T7	TGA CGG AGC AGT GAG AAG	GTT TCC CGC TAC CAA GTC	188
P1-339-C7 SP6	ATG AGG AGG TTT ATC CAG TC	ATC AGC ACC ATT TGA AAT CC	117
P1-339-C7 T7	GTA AGA ACT TAC CAG CCA AGA	TCA CAG CAG GAT GGT TGA AG	153
B722G3 SP6	GTC CTT CCC TAG ACT GCA C	TAT GAA TGA CGC TTC TGG AG	154
B722G3 T7	TTG ATG TGT TGC TGG ATT C	GAT TCA CAG CCG AAC TCT AC	244

[a] The P1-981-C5 SP6, P1-931-D1 T7, and P1-294-F6 SP6 end sequences were repetitive and did not provide good landmarks. Sequencing with P1-1101-F9 T7F, P1-106-A2 T7F, and PAC-25-7K T7F, respectively, confirmed map continuity.

[b] The YAC-derived marker 979H8R was used to identify both P1-546-D4 and P1-1149-C3, confirming continuity.

to establish continuity across the region. Digested P1, BAC, and PAC clones were also screened by hybrid capture using biotinylated repetitive sequence primers to identify new STRs.

Following completion of the P1/PAC/BAC contig, the BRCA2 candidate region was further refined by genetic recombinants in two BRCA2 families. Thirteen polymorphic STRs were examined, 9 of which were new. The primer sequences, products sizes, number of observed alleles, and heterozygosity of these are pre-

TABLE 2

New STR Markers Located in the Region between D1351444 and D13S310

Locus	Primer name	Primer sequence	Average size of STS (bp)	Number of alleles	Het[a]	*n*
D13S1444	tdj3820-R	AATGACTTTATCTACATGAAT	186	9	0.80	70
	tdj3820-LB	CCCTTGCATGGAAAATTGTTAAG				
D13S1700	M4247.4A.2F1	ACCATCAAACACATCATCC	292	18	0.89	76
	M4247.4A.2R2	AGAAAGTAACTTGGAGGGAG				
D13S1699	M4659-SFB	AGACAGAGAATCTCAACTGG	155	6	0.67	78
	M4659-SRB	TTTGATTTTCACAGCAGATG				
D13S1698	YS-GA9-CR1	GTCCATACCACTAAGTCTGAC	177	10	0.63	82
	YS-GA9-CL1	AACCTCAGGCTAATAGTCTCA				
D13S1697	B489G-3C11FB	CTGAAGGTTGGGGTGATTG	221	4	0.47	76
	B489G-3C11RB	CGTAATCCCARCGACTTGA				
D13S1701	MB561A#FA2	GAATGTCGAAGAGCTTGTC	298	8	0.88	74
	MB561A#RB	AAACATACGCTTAGCCAGAC				
D13S1695	M53702C6FA	AGAATCATTGCCCTACTTA	246	11	0.79	78
	M53702C6RA	GATAACTTACCAGCATGTGA				
D13S1696	YS-GB10TR1	CTTCAGAACTTATTAAAGACCTTAG	214	6	0.56	82
	YS-GB10TL1	CTGGTTGTTTTAGAAACTCATAC				
D13S1694	YS-AC6-AAR1	CAATAAGCCCACTTGCGATAC	230	5	0.78	80
	YS-AC6-AAL1	CCATGTGAGGCACCCTGTAAG				

[a] Observed heterozygosity from *n* independent chromosomes.

sented in Table 2. K107 had an affected individual who carried the predisposing haplotype telomeric to *D13S1444,* and K2043 had an affected individual who only carried the predisposing haplotype centromeric to *D13S310.* As shown in Fig. 1, the minimum tiling path across this reduced interval consists of 11 P1s, 6 BACs, and 2 PACs. The contig presented spans approximately 1.8 Mb as two additional BAC clones that extend across the region are also included. The map was consistent with STS content mapping of 51 additional overlapping P1/BAC/PAC clones from this region (data not shown).

Transcribed Sequence Identification

Exon amplification. Three experiments were performed. A total of 6000 clones were picked and gridded for analysis. Prescreening with repetitive DNA and vector DNA eliminated 60–70% of clones. PCR amplification of the remaining 30% revealed that two-thirds did not contain inserts. The final 10% were then analyzed for redundancy and location by hybridization to gridded sets of all candidate exon clones and to blots of genomic DNA from the original cloned genomic DNAs. A total of 75 unique exons were obtained and were sequenced. Twelve exons mapped outside the *D13S1444* to *D13S310* region contained repetitive sequences or were shown to result from cryptic splicing of the vector. Each of the remaining candidate exons was used for cDNA library screening. One or more cDNA clones were isolated for 14 of the 63 candidate exons tested.

Hybrid selection. Hybrid selection was also used to retrieve transcribed sequences from groups of two to four genomic clones, each spanning 250–300 kb as described under Materials and Methods. As the physical map developed, selection was also applied to single genomic clones to ensure that all regions were examined or that regions appearing to be poorly represented in the cDNAs from the grouped clones could be reassessed. Two schemes were employed to analyze the selected cDNA fragments. In the first scheme, hybridization with individual clones was used to determine map position and to provide an assessment of redundancy. The fragments were also verified to originate from chromosome 13 by hybridization to a hamster hybrid containing chromosome 13 as its only human material. Two selected clones were found that hybridized to discrete *Eco*RI fragments in B213E7 DNA and to fragments of corresponding sizes in chromosome 13 DNA but that also hybridized strongly to additional fragments in total human DNA; these were not analyzed further. The remaining 95 of a total of 97 clones were then analyzed by sequencing and used for cDNA library screening. The results for those clones yielding cDNAs are presented in Table 3.

The second scheme of hybrid selection experiments identified a total of 1920 retrieved clones. These clones were screened for the presence of repetitive elements. The remaining nonrepetitive clones were sequenced, condensed according to overlap, and aligned with overlapping sequences from all other putative transcripts in our database. Potential cDNA sequence contigs were also aligned with genomic sequence and scanned for the presence of splice junctions. These sequence contigs revealed unique transcription units or extended the sequences available for identified transcription units. Specific clones from this series were also used to screen cDNA libraries (Table 3).

Genomic DNA sequencing. Genomic DNA sequences obtained by sampling from across the entire

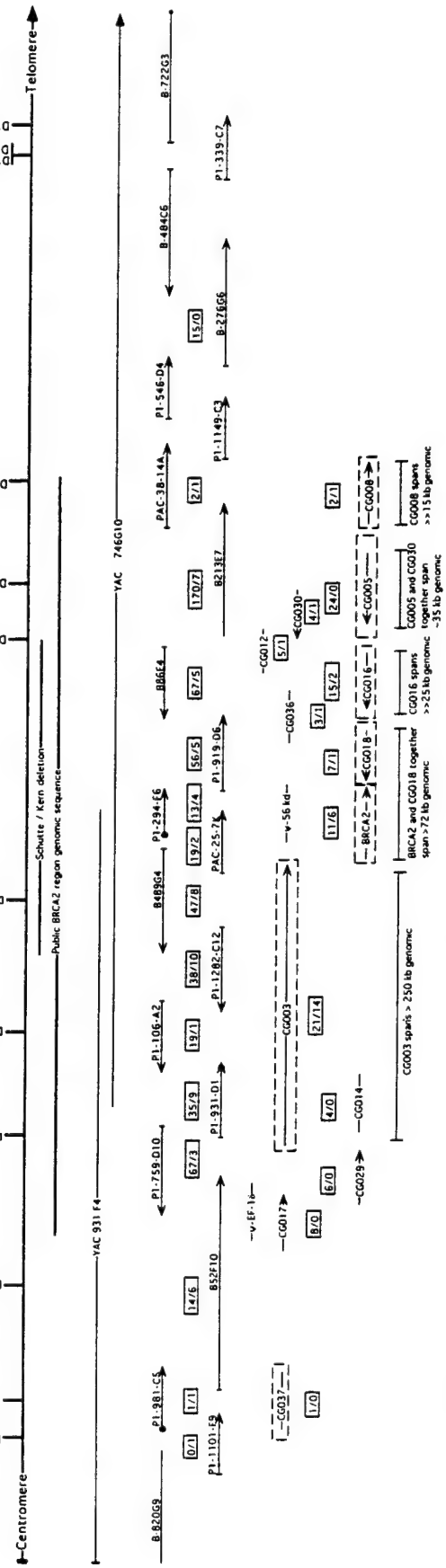


FIG. 1. Integrated genomic/transcript map of the BRCA2 region. The polymorphic loci are shown above the physical map that was assembled from the YACs 931F4 and 746G10 at the *BRCA2* locus. The two polymorphisms at *D13S310* are close together and could not be ordered. The minimal tiling contig of P1, BAC, and PAC clones indicated as horizontal arrows are shown below the YACs. The T7 and SP6 ends are indicated as → and ⊢, respectively. Filled circles indicate clone ends too repetitive to provide useful landmarks. The number of hybrid-selected clone contigs and nonrepeated exon-trapped clones (respectively) that mapped to each P1, BAC, or PAC is indicated by the two numbers in the solid box associated with each genomic clone. Transcribed contigs mapping to overlap regions were counted only once. Transcription units are placed below the P1/BAC/PAC contig. Candidate transcription units are represented by horizontal lines with their name superimposed. Where the orientation of transcription is known, it is indicated by an arrowhead. The Class I transcription units are surrounded by hatched boxes. The subset of hybrid-selected clone contigs and nonrepeated exon-trapped clones, respectively, that mapped to each candidate transcription unit is indicated by the two numbers in the solid box associated with each unit. Alignment of the exons of the transcription units across the genomic sequence allowed a minimum estimate of the genomic intervals spanned by some of the transcription units. These sizes, where known, are indicated below the transcription units.

TABLE 3

Summary of Transcription Units

Transcription unit classes	Probes			Isolated cDNA clones			Transcription units		Remarks
	Name	Size (kb)	Genomic localization	Number of cDNA clones	Size of longest contig (kb)	GenBank Accession No.	Name	Transcript size (kb)	
I	GT563	0.65	B489G4	2	10.7	U50534	CG003	2.1, 3.3, 3.5, 6.5, 8.0, and 11.0	Similarity with *C. elegans* F21H11.2 protein (GenBank U11279)
	GT571	0.6	B489G4	2					
	GT599	0.75	P1-106-A2	2					
	GT601	0.99	B489G4	6					Similarity with *S. cerevisiae* (GenBank P40468)
	GT637	0.4	P1-1282-C12 and B489G4	1					EST D62033, EST H06798, EST H09111, EST H10032
	GT653	0.8	P1-1282-C12	3					EST Z42562, EST H09461, EST D62805, EST H23633
	wXBE1A3	0.18	P1-1282-C12 and B489G4	1					EST H10085, EST F12563, EST H06797, EST T74526
	wXBE1C10	0.27	P1-1282-C12 and B489G4	2					
	wXBE1E11	0.24	P1-1282-C12 and B489G4	1					
	wXPA1B11	0.14	P1-106-A2	1					
	wXPH1H10	0.13	P1-1282-C12 and B489G4	1					
	wXPE1A4	0.12	P1-1282-C12 and B489G4	1					
	GT566	0.9	B231E7	7	2.1	U50532	CG005	2.3, 3.2, 4.4, and 9.5	Similarity with *C. elegans* F26A1.14 protein (GenBank U27312)
	GT575	0.35	B231E7	1					
	GT578	0.6	B213E7	3					Similarity with human 2′, 3′, cyclic nucleotide 3′-phosphodiesterase (GenBank P09543)
	GT607	0.7	B231E7	3					
	GT610	0.55	B231E7	8					
	GT616	0.41	B213E7	10					EST D19650, EST T131302, EST F04257, EST H43430
	GT641	0.45	B213E7	1					EST T36294, EST T11415, EST T59177, EST Z35727
	GT658	0.75	B213E7	1					EST R23446, EST Z39691, EST T10436, EST Z43629
	GT659	0.75	B213E7	8					EST H05696, EST F08013, EST Z37013
	GT568	0.9	B231E7	3	2.3	U50535	CG006		First 82 nucleotides identical to the 3′ end of exon II, while the remaining 2.2 kb correspond to 5′ end of intron II of CG005 gene
	GT603	0.75	B231E7	2					
	GT617	0.75	B231E7	1					EST T70107, EST H86724, EST T70041, EST H92686
									EST H92280, EST T70231, EST H92479, EST Z35727
	GT577	0.5	B231E7	2	1.37	U50536	CG011		Intron III of CG005 gene
	GT576	0.51	B231E7	1	0.68	U50537	CG033		Intron II of CG005 gene, EST H05940
	wXBG1D11	0.19	PAC-38-14A	2	1.85	U50533	CG008	1.4, 5.5, and 8.0	No similarity
	GT642	0.75	P1-294-F6	1	10.9	U43746	CG013 (BRCA2)	0.4, 1.0, and 11.0	Tavtigian *et al.* (1996)
	GT713	0.41	PAC-25-7K	1					
	wXBF1B6	0.3	P1-294-F	1					EST H48122
	wXPF1B8	0.3	P1-294-F6	2					
	WXPF1A5	0.5	P1-294-F6	3					
	GT597	0.6	B86E4	2	2.8	U50529	CG016	4.4	No similarity
	GT660	0.55	B86E4	2					
	GT615	0.6	B86E4	1					
	GT677	0.6	P1-919-D6 and B86E4	1					
	wXPB1F12	0.25	B86E4	1					

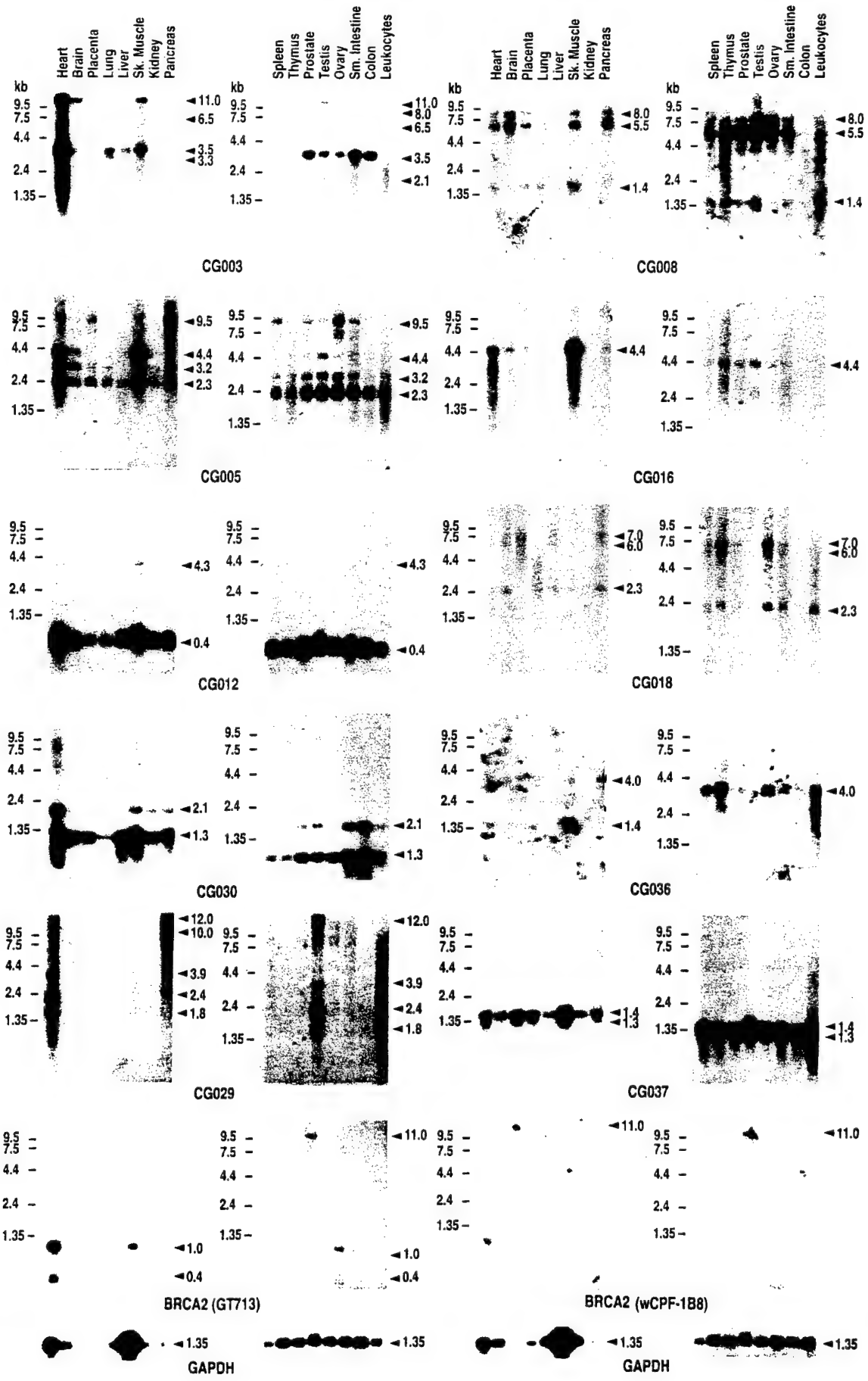
TABLE 3—*Continued*

Transcription unit classes	Probes			Isolated cDNA clones			Transcription units		Remarks
	Name	Size (kb)	Genomic localization	Number of cDNA clones	Size of longest contig (kb)	GenBank Accession No.	Name	Transcript size (kb)	
	GT711	0.75	P1-919-D6	1	2.05	U50527	CG018	2.3, 6.0, and 7.0	EST R61510, EST R41970, EST F03825, EST Z38606 EST F02671
	wXBF1A6	0.25	P1-919-D6	1					
	GT627	0.4	B52F10	21	1.43	U50523	CG037	1.3 and 1.4	EST R69912, EST T33994, EST H74118, EST R63793 EST R81762, EST R64207
II	GT565	0.45	B86E4	6	1.42	U50530	CG012	0.4 and 4.3	Colinear with genomic sequence
	GT661	0.38	B86E4	9					EST H18362
	Hexp17.1E05	0.6	P1-759-D10	5	1.62	U50525	CG029	1.8, 2.4, 3.9, 9.5, and 12.0	Colinear with genomic sequence, no similarity
	Hexp10.2F08	0.32	B213E7	1	4.9	U50531	CG030	1.3 and 2.1	Colinear with genomic sequence
	wXBG1C7	0.15	B213E7						EST L44472
	GT574	0.41	P1-919-D6 and B86E4	1	0.95	U50528	CG036	1.4 and 4.0	Colinear with genomic sequence No similarity
III	GT572	0.6	P1-931-D1	1	1.2	U50526	CG014	ND	Colinear with genomic sequence, no similarity
	Hexp12.1G01	0.7	P1-759-D10	1	2.3	U50524	CG017	ND	Colinear with genomic sequence, no similarity
	GT573	0.75	P1-931-D1	3	0.75	U50538	—	ND	Colinear with genomic sequence, no similarity
	GT605	0.5	B213E7	1	0.5	U50539	—	ND	Colinear with genomic sequence, no similarity
	GT650	0.75	B276C6	2	0.45	U50540	—	ND	No similarity
IV	—	—	P1-759-D10 and B52F10	—	—	—	ψ-EF-1 δ	ND	Putative pseudogene: altered form of transcription elongation factor-1d (TEF-1D)
	—	—	P1-294-F6	—	—	—	ψ-56 kDa	ND	Putative pseudogene: non-coding copy of interferon inducible 56-kDa protein (GenBank Accession No. M24594)

Note. A summary of the retrieved clones and the corresponding isolated cDNA is listed. The clones are grouped according to the transcription units shown in Fig. 1. The longest cDNA "contig" or "contigs" of each transcription unit as given were obtained by compiling overlapping sequences of selected or trapped clones and their corresponding cDNAs obtained by library screening. The contig sequence was assigned a GenBank Accession number as indicated.

interval facilitated the merging of groups of transcribed contigs and revealed transcription unit organization and genomic structure. Sequences of P1, BAC, or PAC clones were obtained with oligonucleotides corresponding to clone vector ends or from internal oligonucleotides designed from selected or trapped transcribed sequences. Additional sequences were obtained from shotgun cloning of small fragments of the larger genomic clones. In total, 30% of the sequence of the region from BAC B52F10 to PAC-38-14A was obtained. cDNA contigs generated by exon trapping, hybrid selection, and cDNA library screening were aligned with the genomic sequences to help identify splice junctions and resolve issues of transcription unit organization. However, with this limited amount of genomic sequence, spatial relationships between the retrieved transcribed contigs proved difficult to establish even when the genomic clones of origin were known. During the course of this work 900 kb of genomic sequences from the interval outlined in Fig. 1 became publicly available from the Sanger and Washington University sequencing centers. These genomic sequences were aligned with our genomic sequences and could be merged into a set of 160 sequence contigs.

Assembly of transcription units. Contigs of transcribed sequences were grouped into tentative transcription units based on (1) map position, (2) overlap of sequence following isolation of longer cDNA clones from cDNA libraries that provided for extension of contigs or for merging of small contigs, (3) recognition of poly(A) tails and corresponding polyadenylation signals, and/or (4) RNA hybridization analysis. The results are summarized in Fig. 1 and Table 3. Identities to public ESTs are also indicated in Table 3. The RNA hybridization results are shown in Fig. 2. In total, four classes of transcription units were assigned. The first class included seven transcription units or genes for which complete (three) or partial transcription contigs


Heart
Brain
Placenta
Lung
Liver
Sk. Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Sm. Intestine
Colon
Leukocytes
kb
CG003
CG008
CG005
CG016
CG012
CG018
CG030
CG036
CG029
CG037
BRCA2 (GT713)
BRCA2 (wCPF-1B8)
GAPDH

(four) were isolated, each with putative extended coding potential. Furthermore, these contigs aligned with genomic sequence such that gene structure with exons and introns could be recognized following alignment. Each of these genes, including CG003, CG005, CG008, CG013 (BRCA2), CG016, CG018, and CG037, detected discrete transcripts in human tissues as shown in Fig. 2 and will be described individually below.

The second class included the four transcription units CG012, CG029, CG030, and CG036, for which extended cDNA sequence was obtained from isolated cDNAs. Their respective expression profiles in a variety of human tissues were distinct and thus suggest that they correspond to discrete transcription units (Fig. 2). Further, their physical position indicated dispersal across the interval and thus also supported the likelihood of each being a discrete unit. However, all of these transcript contigs appeared to be colinear with genomic DNA and did not appear to possess extended coding capacity. Whether these units encoded polypeptides was therefore uncertain.

During the characterization of CG012, it appeared that some isolated cDNAs did not align precisely to the genomic DNA sequence. It was not possible to establish that these cDNAs were actually transcribed from the chromosome 13q12–q13 region. That a gene family was involved appeared reasonable but also limited the conclusions that could be drawn from the observed expression in the human tissues even though it was known that the cDNA that was used for hybridization to RNA was verified to originate from chromosome 13.

The third class of transcription units included sets of contigs built largely by exon or hybrid selected clones with longer cDNA clones. They differed from class II units in that no RNA hybridization analysis was performed. They included the units designated CG014, CG017, GT573, GT605, and GT650 in Table 3. From the limited sequence that was obtained, four appeared to be colinear with genomic sequence. GT650 could not be analyzed as no genomic sequence from B276G6 was available. This class of transcription unit may correspond to genes that code for polypeptides, but insufficient information was obtained to establish this.

The fourth class included two transcription units for which evidence was obtained to suggest that they were pseudogenes. The genomic sequence revealed frameshift alterations in a sequence nearly identical to the gene for a transcription elongation factor (TEF-1d) in the overlap interval of B52F10 and P1-759-D10. Two hybrid selected clones corresponding to this sequence were obtained, one with identity to the reported gene sequence and one with perfect alignment to the genomic sequence with internal frameshifts. The second pseudogene aligned with the gene for an interferon-inducible 56-kDa protein. This sequence also contained frameshifts when compared to the GenBank entry of the original gene (Accession No. M24594) and was positioned in intron 24 of the BRCA2 gene.

In summary, a total of 27 of the 63 trapped exons were accounted for by sequence alignment to transcription units. The numbers of exon clones isolated from specific genomic clones and those present in the individual transcription units are indicated in Fig. 1. The other 36 putative exons are spread across the entire region with a noted number of 6 from the B52F10 BAC genomic clone alone. A total of 563 hybrid-selected clone contigs are shown on the map in Fig. 1, 111 of which are contained within the 7 candidate genes and the transcription units as indicated. Similarly, as with exon-trapped clones, many of the hybrid selected clones are not contained within the transcription units from Table 3. The organization of transcription units provided some insight into the distribution of these nonassembled transcribed sequences.

Organization of transcription units. Together with the sequence generated during the course of this work and with the genomic sequence made publicly available, each candidate gene or transcription unit could be aligned. Additional information including direction of transcription, an estimate of the minimum size of the corresponding genomic region, and relative proximity (Fig. 1). This exercise also enabled the ordering many of the independent sequence contigs across the region, though individual contigs completely within a gap or an interval of nonoriented sequence would have been missed.

The interval between CG037 and CG003 spans more than 100 kb. It contains one Class II transcription unit (CG029) and one Class III transcription unit (CG017) as well as singlet exon-trapped and hybrid-selected clones. The genomic clone B52F10 was not used in a second round of hybrid selection, and only a limited amount of gene assembly was feasible. Given the number of nonassembled exons that were found to map to B52F10 (see previous section), there is a strong possibility that the interval contains unidentified genes.

CG003 spans at least 250 kb of genomic sequence. The first intron of CG003 spans more than 29 kb, and the apparent inclusion of the Class III transcription unit CG014 within the intron is noteworthy. It was not possible to establish whether CG014 did contain equivocal coding capacity as the sequence that was

FIG. 2. Hybridization of cDNAs to poly(A)$^+$ RNA. Representative clones of 11 transcription units are shown hybridized to 2 μg of poly(A)$^+$ RNAs of the tissues indicated. The sizes of the markers listed on the left and the sizes of mRNAs, as indicated by the arrows on the right, are given in kilobases (kb). As control for the amount of RNA present in each lane, the blots were hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment. Increased levels of RNA in heart and skeletal muscle relative to other tissues are apparent. Blots were exposed to Kodak X-Omat AR film with one intensifying screen at −70°C. 0.5 to 10 days were needed to optimize signal.

available was limited. This transcription unit may correspond to an intron sequence as retrieval of transcribed but unspliced sequence by either hybrid selection or cDNA library screening is plausible. However, of the 28 unique trapped exons that mapped to the region encompassing CG003, only 14 corresponded to CG003 itself and 9 distinct exons mapped to within the P1-931-D1 genomic clone. While cryptic splicing artifacts can be generated, it is unlikely that so many would be isolated from a single physical interval. Further, based on RNA hybridization, these exons did not correspond to the large or alternative transcripts seen for CG003 (data not shown). These results together suggest the possibility of at least one additional gene in this physical interval.

The 3′ end of CG003 and the 5′ end of BRCA2 both map to the overlapping interval of B489G4 and PAC-25-7K. The alignment of end exons of CG003 and BRCA2 reveals that the intervening region contained approximately 24 kb of genomic sequence. The positioning or order of 9 hybrid selected clones (2 of which overlap to form a contig) is restricted to the region of the 3′ introns of CG003, the 5′ introns of BRCA2, and the region between the 2 genes. In contrast, 24 kb of genomic sequence, including the 5′ end of intron 11 of BRCA2 extending into intron 24, contained 48 hybrid selected clones that were assembled into 9 contigs and 13 singlets (Note that only the contigs are indicated in Fig. 1.) In addition, the putative introns did not give rise to trapped exons. Taken together, it appears unlikely that any additional genes occur between CG003 and BRCA2. This evaluation was feasible as the genomic sequence was nearly complete in this region. Further, given that BRCA2 is not an abundantly expressed gene (Tavtigian *et al.,* 1996; Fig. 2), the depth of detection of transcribed sequences by the exon trapping and hybrid selection procedures was adequate. The majority of exons and transcribed contigs from the region were explained as *bone fide* BRCA2 sequences.

Although the exact polyadenylation site of BRCA2 is unknown, the available and converging 3′ ends of BRCA2 and CG018 are spaced by only 2 kb. CG018 and CG016 are transcribed in the same direction and map to the overlapping genomic clones P1-919-D5 and B86E4, respectively. Comparison of the size of their longest cDNA contig with their transcript sizes estimated from Northern blots reveals that neither of these genes has been entirely cloned. In addition, the Class II transcription unit CG036 plus a large number of hybrid selected clones that have not been assigned to any named transcription unit map between them. It is likely that additional exons of CG016 or CG018 are also present.

CG016 and CG005, which are also transcribed in the same direction, map to the overlapping genomic clones B86E4 and B213E7, respectively. The polyadenylation signal of the CG005 splice isoform encoding the longest putative open reading frame currently identified lies 11 kb telomeric of the SP6 end of B86E4, in B213E7. However, genomic sequences across CG016 are somewhat sparse, so the distance from the 5′ end of CG016 to the SP6 end of B86E4 is unknown. A very large number of hybrid-selected clones, almost all transcribed in the same direction as CG005, were captured from the 11 kb of genomic sequence immediately downstream of the CG005 polyadenylation signal. The sequence of the Class II transcription unit CG030 was assembled from only some of these clones. A second Class II transcription unit, CG012, also maps between CG005 and CG016. CG030 and CG012 may be representatives of *bona fide* genes lying between CG005 and CG016. Alternatively, they may be included in introns or be a part of a larger transcript that includes both CG005 and CG016 in a single transcription unit. This latter possibility has not yet been ruled out and is supported by the common 4.4-kb transcript that was seen in RNA of several tissues with representatives of each of these two assigned units (Fig. 2).

A large number of hybrid-selected clones plus 7 exon-trapped clones map to the interval between the currently known 5′ ends of CG005 and CG008. Genomic sequences across CG008 were very sparse, so only a limited amount of alignment was feasible, thus restricting additional analysis.

Characterization and expression patterns of transcription units. The analysis of the sequence of each of the transcription units and the RNA survey analysis of a range of human tissues provided for the identification and characterization of 6 new genes. CG013 corresponds to BRCA2 and is positioned essentially in the center of the region analyzed. Its partial (Wooster *et al.,* 1995) and complete (Tavtigian *et al.,* 1996) putative amino acid sequence has been reported. The structural organization of the BRCA2 gene, which consists of 27 exons spanning 60–70 kb, has also been described (Tavtigian *et al.,* 1996). This gene was initially merged from 3 candidate transcription contigs with the identification of the large open reading frame of a 4.9-kb exon from the genomic DNA sequence. cDNAs corresponding to this gene were identified from a subset of 6 trapped exons and 11 hybrid-selected clones, and the final sequence was obtained by overlap consensus. The expression of this gene as determined by RNA hybridization and RT-PCR has been described (Tavtigian *et al.,* 1996). Hybridization of three different cDNA probes to human multiple tissue Northern filters revealed an 11- to 12-kb transcript that was detectable in testis, thymus, and placenta (Tavtigian *et al.,* 1996, Fig. 2) thus suggesting that little, if any, of the BRCA2 mRNA sequence is missing from our composite cDNA. As illustrated in Fig. 2, smaller transcripts were also detected in several tissues by using the cDNA probe GT713, which corresponds to BRCA2 exons 3–7, but were not detected with the probe wCPF1B8.1, corresponding to the 3′ end of exon 11 through to exon 20. The significance of these smaller transcripts is unclear.

CG037, present on P-1981-C5, is the most centro-

meric of the genes identified (Fig. 1). This gene was abundantly expressed as a doublet mRNA of 1.3 and 1.4 kb in all 16 human tissues tested (Fig. 2). Twenty-one cDNA clones were obtained by screening with a single hybrid-selected clone. The compiled sequence of the overlapping cDNAs revealed the presence of a long open reading frame of 248 amino acids with 5′ and 3′ untranslated regions of 248 and 444 nucleotides, respectively. Database analysis indicated alignment with 45 different ESTs but not to any known genes at either the nucleotide or the amino acid sequence level. Only the six ESTs having greater than 90% identity over >300 nucleotides are indicated in Table 3. It is of interest that, in contrast to the 1.4-kb transcript that is expressed in all tissues surveyed, the second, and smaller, mRNA species was not detectable or was weakly expressed in several tissues. The nature of the difference between the transcripts was not explained from the sequencing analysis but likely results from tissue-specific alternative splicing or polyadenylation.

A large gene, CG003, spanned over 250 kb of genomic DNA. RNA hybridizations revealed two prominent transcript sizes of 11.0 and 3.5 kb. The larger message, which is composed of 61 exons, was the only one expressed in the brain but was broadly expressed together with the 3.5-kb mRNA in other tissues including heart, skeletal muscle, testis, placenta, pancreas, spleen, thymus, prostate, ovary, colon, and small intestine. Complete sequence of cDNAs corresponding to 10.7 kb was obtained. The amino acid sequence aligned with significant similarity to extended open reading frames that have been identified in both *S. cerivisiae* and *C. elegans* genomic sequencing efforts, but only 12 human ESTs have been reported that could be aligned. The function of the protein encoded by this gene is not known. This gene is clearly complex as hybridization with GT601 (nucleotides 9461 to 10460 of the composite cDNA) revealed additional transcripts of 8.0 and 2.1 kb in testis RNA and a prominent mRNA of 6.5 kb in the kidney. Further, only mRNAs of 3.5 and 3.3 kb were present in the lung. The mRNA of 3.5 kb that generally appeared most prominent was absent or expressed at low levels in placenta, kidney, spleen, and thymus. The doublet 3.5- and 3.3-kb transcript and the 9.5-kb transcript were also observed in the HepG2 cell lines by using GT601 as probe but only the 9.5-kb mRNA species was detectable when GT653 (nucleotides 4807 to 5657 of the composite cDNA) was used as probe (data not shown). The complete alignment and the hybridization of the probes GT601 and GT653 to genomic DNAs confirmed their origin as the BRCA2 region. Additional experiments will be required to delineate the relation of these multiple transcripts and to obtain a better assessment of the large message. Its expression pattern was partially compromised by the varying abundance of mRNAs present on the RNA blots used; see control (GAPDH) samples in Fig. 2.

The gene CG018 involved up to three mRNAs of 7.0, 6.0, and 2.3 kb as determined by using wXBF1A6 (nucleotides 231 to 448) as a probe. The larger pair of transcripts and the smaller transcript demonstrated distinct but restricted expression profiles as shown in Fig. 2. Only a portion of this gene was recovered, but an extended segment with good coding capacity and aligned consensus splicing signals were present. Alignment to the public databases was restricted to five ESTs.

CG016 was characterized by a restricted pattern of expression with a 4.4-kb mRNA being most abundant in heart, brain, skeletal muscle, thymus, prostate, testis, ovary, and small intestine. The cDNA probe used, wCPB1F12.T8, corresponds to nucleotides 451 to 902. Seven cDNAs were isolated with hybrid-selected clones and a single exon-trapped clone to yield a partial contig of 2.2 kb. This limited sequence was found to span more than 25 kb of genomic DNA and revealed the presence of a long putative open reading frame of 635 amino acids with 5′ and 3′ untranslated regions of 323 and 583 nucleotides, respectively. No similarities were identified in the databases.

A composite cDNA sequence of 2.1 kb was found for CG005. The sequence was assembled from 42 cDNA clones and contains a long open reading frame of 583 amino acids with 5′ and 3′ untranslated regions of 169 and 197 nucleotides, respectively. The predicted open reading frame is derived from six exons. Sequence analysis indicated similarity to 15 ESTs and a portion of the putative coding region revealed a good alignment with the *C. elegans* F26A1.14 protein and to human 2′,3′ cyclic nucleotide 3′ phosphodiesterase over 177 amino acids. This gene is relatively complex as four different transcripts with lengths of 9.5, 4.4, 3.2, and 2.3 kb were detected by RNA blot analysis using GT616 (nucleotides 891 to 1301) as probe. All tissues displayed the smaller mRNA with several tissues expressing different combinations of the larger messages as shown in Fig. 2. Three additional transcribed contigs have been mapped to the genomic region containing CG005, as listed in Table 3. They include CG006, CG011, and CG033. Their role in the CG005 transcript remains to be precisely determined, although it was noted that the sequence of each of these contigs was a colinear genomic sequence and they are all transcribed in the same direction as CG005. Thus, they may correspond to products of hnRNA. CG006 included exon II and intron II boundaries of CG005 and has been reported as an EST.

The most telomeric Class I transcription unit, CG008, was derived from one exon and two hybrid-selected clones. Two cDNAs were isolated using the exon-trapped clone to yield a final transcribed composite contig of 1.8 kb. Three transcripts of 8.0, 5.5, and 1.4 kb were detected in most human tissues analyzed but relative levels varied considerably as shown in Fig. 2. Transcript analysis was carried out with wCBG.1D11#2, which corresponds to nucleotides 1 to 1066 of the composite cDNA. No similarity to known genes or proteins was observed despite the presence of

an extended segment with good coding capacity of 384 amino acids.

The other transcription units derived from hybrid-selected clones and cDNA library clones belonging to Classes II and III are presented in Table 3. Their composite sequences (with the possible exception of GT650) are colinear with genomic sequence. No extended open reading frames or consensus splice sites were identified within these sequences. No similarity with other sequences has been observed with the exception of single ESTs showing sequence identity with CG012 and CG030. Northern blot analysis has been performed for CG012, CG029, CG030, and CG036 and revealed distinct tissue-specific expression profiles (Fig. 2). CG030 is composed of a 4.9-kb contig derived from a single cDNA clone, 4 hybrid-selected clones, and 1 exon-trapped clone. Expression analysis identified two transcripts of 1.3 and 2.1 kb. The disparity between the expression data and the cDNA sequence data together with the absence of splice signals suggested that the 4.9-kb contig may contain chimeric cDNA cloning artifacts. It was noted that CG018, CG036, and CG016 are all located in a region of less than 100 kb; however, RNA hybridization analysis clearly reveals independent transcripts. GT573 is a transcription unit located in P1-931-D1 within an intron of the BRCA2 gene. GT573 consists of a 0.75-kb contig composed of three cDNA clones from cDNA libraries and the original hybrid-selected clone. GT573 has no significant homology to any previously described cDNA sequence.

CONCLUSIONS

Genome sequencing of prokaryotic genomes or whole chromosomes of *S. cerevisiae* or *C. elegans* has produced, as a matter of course, extended transcript maps. In contrast, extended human transcript maps are less detailed but have appeared most advanced where positional cloning projects of diseases or conditions have demanded the refined integration of physical data and candidate gene characterization. As these projects are driven by the endpoint set by the disease being studied, less emphasis is placed on the completion of the local transcription map. There are also the limitations caused by the lack of complete and oriented genomic sequence information or of the ability to analyze adequately the long stretches of DNA. The largest gene that was identified in this study, also corresponding to the largest mRNA and extending over 250 kb of genomic DNA, displayed strong similarity to putative open reading frames identified by the *C. elegans* and *S. cerevisiae* genomic sequencing projects. The extensive open reading frame of *C. elegans* spanned much less genomic distance and was used as a guide to complete the cloning of the entire long transcript of the human gene. When the genomic sequence of this human gene becomes polished, it will be interesting to compare the effectiveness of gene detection software in the human genomic sequence versus model organism genomic sequence. Further, this test case would provide for an evaluation of the extent of the complexity of genes that could be revealed based solely on the analysis of genomic sequence (i.e., can the relation of the additional and alternate sized transcripts be revealed?).

Although the retrieval of transcribed segments involves common procedures, the more challenging aspect of building transcription maps includes the assembly of retrieved sequences into transcription units. While the assembly in this study was supported by the generous amount of genomic sequence, extensive effort was still needed to verify contiguity of transcripts. This was, at least partially, a result of having observed several large and/or alternatively spliced transcripts. The genomic sequence did reveal that two transcription units identified were pseudogenes. A very serious limitation was noted with the restricted genomic sequencing initially performed. The long open reading frame of the large exon of the BRCA2 gene was missed by our first analysis and not recognized until more genomic sequence became available.

An integrated analysis of the transcribed sequences of the interval between *D13S1444* and *D13S1695* revealed a series of at least seven transcription units with extended and substantial coding potential, two pseudogenes, and at least nine additional transcription units as revealed by identification of short transcribed segments and RNA hybridization. Precise location for each transcription unit could be established from the refined physical map achieved with the overlapping P1, PAC, and BAC genomic clones. Evidence was obtained that the analysis was incomplete and that additional transcription units were likely to be present, as a large number of putatively transcribed contigs and a number of trapped exons did not fit into the assigned transcription units. The refined analysis of the region at and near the BRCA2 gene indicated that the majority of the transcribed contigs could be assigned if genomic sequence was available. Some of the transcription units simply may not have been characterized sufficiently to find their open reading frames. Efforts to attempt to link the transcribed contigs or exons, especially for those that appeared to group within short physical distances of each other, would aid in clarifying their existence. Anticipated completion of the genomic sequence by the Sanger and Washington University sequencing centers in combination with the transcription maps that were generated to find BRCA2 candidates will provide a more complete picture of the transcribed sequences at the *BRCA2* locus.

At least four genes, CG016, CG018, BRCA2, and a portion of CG003, are deleted in a pancreatic carcinoma that has been reported (Schutte *et al.,* 1995a,b). While involvement of BRCA2 in the development of pancreatic cancer is reasonable, additional roles for these other genes cannot be excluded.

ACKNOWLEDGMENTS

B.L.W. is supported by NIH Grants CA57601 and CA61231. F.J.C. is supported by NIH grant CA67403. J.M.R. and J.S. are Scholars

of the Medical Research Council (MRC) of Canada while F.L. is a MRC Career Scientist. J.S. and F.L. are supported by MRC and Endorecherche Inc. S.N., M.H.S., and D.E.G. are supported by U.S. Army Grant DAMD17-94-J-4260 and NIH Grants CA55914, CA48711, CA65673, RR00064, CN05222, and CA42014. J.M.R. is a member of the Canadian Genetic Diseases Network.

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Recurrent *BRCA2* 6174delT mutations in Ashkenazi Jewish women affected by breast cancer

Susan Neuhausen[5], Teresa Gilewski[3], Larry Norton[3], Thao Tran[5], Peter McGuire[1], Jeff Swensen[5], Heather Hampel[1], Patrick Borgen[4], Karen Brown[1], Mark Skolnick[6,7], Donna Shattuck-Eidens[6], Suresh Jhanwar[2], David Goldgar[7] & Kenneth Offit[1]

The lifetime risk of breast cancer may approach 80–90% in women who have germline mutations of either of two genes, *BRCA1* or *BRCA2* (refs. 1–3). A single *BRCA1* mutation, 185delAG, has been noted in approximately 20% of Ashkenazi Jewish women with early onset breast cancer and in 0.9% of the Ashkenazi population[4–6]. We recently detected a 6174delT frameshift mutation in *BRCA2* in an hereditary breast cancer kindred of Ashkenazi Jewish ancestry. Here, we investigated the frequency of this mutation in 200 women with early-onset breast cancer. Six of 80 Ashkenazi Jewish women (8%) diagnosed with breast cancer before the age of 42, were heterozygous for the 6174delT mutation, compared to none of 93 non-Jewish women diagnosed with breast cancer at the same age ($P = .005$). These cases were ascertained without regard to family history. Two of 27 (7%) additional Jewish families in which the proband was diagnosed with breast cancer at age 42 to 50 and had a family history of breast or ovarian cancer had germline 6174delT mutations. The results of this report suggest that a recurrent mutation of *BRCA1* and a recurrent mutation of *BRCA2* together may account for over a quarter of all early-onset breast cancer cases and two thirds of early-onset breast cancer in the setting of a personal or family history of ovarian cancer in Ashkenazi Jewish women.

[1]*Clinical Genetics Service, Department of Human Genetics,* [2]*Cytogenetics Service,* [3]*Breast Oncology Service, Department of Medicine, and* [4]*Breast Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York New, York, 10021, USA* [5]*Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah, 84108, USA* [6]*Myriad Genetics, Inc., Salt Lake City, Utah, 84108, USA* [7]*Unit of Genetic Epidemiology, International Agency for Research on Cancer, 150 cours Albert Thomas, 63972, Lyon, Cedex 08, France*

Correspondence should be addressed to K.O.

Mutations in the *BRCA2* gene are responsible for approximately half of hereditary breast cancer[2,7]. Women with *BRCA2* mutations appear to have the same breast cancer risk as *BRCA1* mutation carriers, and, although not as high as in *BRCA1*-linked kindreds[2], are also at an increased risk for ovarian cancer. We have recently reported the complete coding sequence and genomic structure of *BRCA2*, and described nine mutations in addition to the six originally reported by Wooster *et al.*[7,8]. One of these mutations, a single base pair deletion in codon 1982 of exon 11, 6174delT, was identified in an individual of Ashkenazi Jewish ancestry. This mutation creates a termination codon at 2003.

To assess the frequency of the 6174delT mutation in Ashkenazi women with early onset breast cancer, we tested samples serially ascertained at Memorial Sloan-Kettering Cancer Center. Specifically, 80 women of Ashkenazi Jewish ancestry with breast cancer before the age of 42 were compared to a control group of 93 non-Jewish women who had breast cancer before the age of 42. These cases were ascertained without regard to family history.

The *BRCA2* 6174delT mutation was observed in 6 of 80 cases (8%) [95% Confidence Interval (C.I.) 2–13%] of breast cancer in Jewish women diagnosed before age 42 (Table 1, group 1b), compared to none of 93 non-Jewish women diagnosed with breast cancer at the same age (group 1a) ($P = .005$, Poisson test). This mutation was also not detected in 70 controls with no history of cancer. In each of two kindreds where multiple samples were available for analysis, the 6174delT mutation co-segregated with two or more cases of breast or ovarian cancer. Four of the six cases with the 6174delT mutation had a family history of breast or ovarian cancer. Nine cases in group 1b had a personal or family history of ovarian cancer. Of these, one (11%) had a *BRCA2* 6174delT mutation.

A second cohort (group 2) of 27 Ashkenazim with breast cancer at age 42–50 and a history of at least one first or second degree relative affected with breast or ovarian cancer provided an additional estimate of the frequency of the 6174delT mutation. In this group of 27 women, 2 (7%) [C.I. 1–18%] were heterozygous for the *BRCA2* 6174delT mutation. One of these individuals had first degree relatives with both ovarian and breast cancer.

When the data here are combined with prior data on the frequency of the 185delAG *BRCA1* mutation in this cohort[6], 22 women from group 1b had either a 185delAG *BRCA1* or a 6174delT *BRCA2* mutation, and, of these, 7 (32%) had a personal or family history of ovarian cancer. In both groups 1b and 2, 12 of 32 women (38%) with either a 185delAG *BRCA1* or a 6174delT *BRCA2* mutation had a family or personal history of ovarian cancer. This compares to 2 of 58 (3%) Jewish women in group 1b and 6 of 75 (8%) Jewish women in groups 1b and 2 combined, with neither of these mutations and a similar history of ovarian cancer ($P = .005$ and $P = .006$, respectively).

Table 1 Analysis of *BRCA2* for presence of the 6174delT mutation in breast cancer patients

Group	Subjects tested	Subjects with 6174delT	(%)
Group 1a			
Diagnosis before age 42 Non-Jewish[a]	93	0	(0)
Group 1b			
Diagnosis before age 42 Jewish[a]	80	6	(8)
before age 37	40	4	(10)
age 37–41	40	2	(5)
Group 2			
Diagnosis ages 42–50 and family history positive[b]	27	2	(7)
Controls[c]	70	0	(0)

[a]Ascertained regardless of family history.
[b]Family history for this group was defined as one first degree or two second degree relatives diagnosed with breast or ovarian cancer, one before age 50.
[c]Controls were Caucasians unaffected by cancer.

Fig. 1 Acrylamide gel analysis of the 6174delT *BRCA2* mutation in 19 Ashkenazi Jewish individuals with breast cancer. Samples labeled 2 and 63 are heterozygous for this mutation.

Overall, the two mutations, 185delAG of *BRCA1* and 6174delT of *BRCA2*, accounted for 22 of 80 (28%) [95% C.I. 18–37%] cases of early onset (before age 42) breast cancer in Ashkenazi Jewish women. By contrast, the prevalence of all *BRCA1* mutations documented in over 100 women with early-onset breast cancer was 7–8% (refs 6,9). However, an analysis of *BRCA2* mutations was not performed in these reports. In addition, in this series, 185delAG or 6174delT mutations were observed in two-thirds (12 of 18) of Ashkenazi Jewish individuals with early onset breast cancer who had a personal or family history of ovarian cancer.

While all of the *BRCA1* 185delAG heterozygotes identified in our prior analysis of this cohort[6] had a history of a first or second degree relative with breast or ovarian cancer, no such history was noted in 2 of the 6 *BRCA2* 6174delT heterozygotes among the 80 women ascertained solely on the basis of early-onset disease. These findings may be due to transmission of the gene through males, chance segregation or to a lower penetrance than predicted. From groups 1b and 2, among 53 families with at least two cases of breast cancer in first degree relatives with one breast cancer case diagnosed on or before age 50, 17 (32%) demonstrated a germline *BRCA1* 185delAG mutation[6], and 4 (8%) a *BRCA2* 6174delT mutation. Thus, a significant proportion of early-onset hereditary breast cancer in Ashkenazi women is due to other mutations of *BRCA1* or *BRCA2*, to mutations of other genes as yet uncharacterized, or to chance aggregations. These considerations should be included in the counseling of Jewish women seeking testing for the 185delAG or 6174delT mutations, since the predictive value of a negative test for these two mutations will be low. Furthermore, because the cohorts in this study were selected on the basis of age of onset or family history, the results cannot be extrapolated to the general Ashkenazi Jewish population.

From the data in this report, and assuming a penetrance similar to *BRCA1* mutations, the frequency of the 6174delT mutation in Ashkenazim can be estimated to be approximately 3 per 1,000. However, if the penetrance of this mutation is lower than *BRCA1*, then the frequency of this mutation will be higher. A more precise estimate of the carrier frequency of the 6174delT mutation in individuals of Ashkenazi Jewish ancestry will emerge from large-scale population studies.

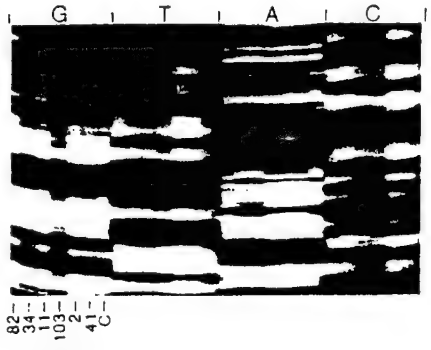

Fig. 2 The 6174delT mutation. Sequencing reactions (ordered G, T, A and C for each sample) were loaded side by side. The primers were CGORF-RH and TD-SFB with sequencing using the CGORF-RH primer. Lanes 1–6, Ashkenazi Jewish women with breast cancer; lane 7, control. Samples 82, 103, 2 and 41 are heterozygous for the *BRCA2* 6174delT mutation and confirm the PCR analysis.

Methods

Ascertainment. Two groups of probands comprised the ascertainment for this study. The first group (group 1b, Table 1) was ascertained solely by age-of-onset of breast cancer without regard to family history. The second group (group 2, Table 1) was ascertained based on both age-of-onset and a positive family history. The first group consisted of probands affected with breast cancer on or before 41 years of age with or without a family history of breast cancer. Inclusion criteria for the second group were: the proband was affected with breast cancer between the ages of 41 and 51 with one or more first degree relatives affected with breast or ovarian cancer on or before the age of 50; or the proband was affected with breast cancer between the ages of 41 and 51 with two or more second degree relatives affected with breast or ovarian cancer, 1 on or before age 50; or the proband was affected between the ages of 41 and 51 with both primary breast and primary ovarian cancer. Probands for the first group were ascertained primarily through medical oncology clinics, while families for the second group were ascertained primarily through a referral genetic counseling clinic. The group of 80 Jewish probands was drawn from 395 patients who indicated a religious affiliation at time of enrollment in a research protocol. Participation in this study was offered to each individual meeting eligibility criteria during the three and a half year period of the study. Family history was obtained by a self-report questionnaire. Histologic confirmation of diagnosis was obtained for probands in all cases. Religious background was confirmed on all probands by self report or interview. All individuals gave informed consent for genetic studies as part of institutional review board-approved protocols. Those individuals indicating interest in receiving *BRCA1* or *BRCA2* mutation results as part of a second stage of consent will first undergo confirmation of results by sequencing of a second sample, and will be informed of these results in the context of genetic counselling[10]. Two sets of controls were utilized. The first consisted of 93 non-Jewish patients with breast cancer diagnosed before the age of 42, randomly drawn from the group of 315 non-Jewish patients that constituted the ascertainment for group 1a. A second group of controls consisted of 70 Americans of European descent with no history of cancer, ascertained at the University of Utah.

Estimate of the 6174delT carrier frequency (q). This was performed by: 1) assuming a relative risk (R) of developing breast cancer before age 42 in 6174delT heterozygotes of 30 (compared to all non-carriers); 2) fixing the proportion of breast cancer cases in the Ashkenazi Jewish population attributable to the 6174delT at the value observed in the present study (O); 3) solving the equation relating the frequency, penetrance, and attributable risk parameters, $O=Rq/(Rq+1-q)$, for q.

Mutation detection. The *BRCA2* 6174delT mutation was detected by acrylamide gel electrophoresis (AGE). Amplification of genomic DNA was performed according to standard PCR procedures[11–13] with an annealing temperature of 55 °C. A total reaction mix of 10 ul containing 20 ng of DNA was utilized with the following primers: BC11-RP: 5'–GGGAAGCTTCATAAGTCAGTC–3', BC11-LP: 5'–TTTGTAATGAAGCATCTGATACC–3'

The PCR product sizes were 87 bp for the non-deleted segment and 86 bp for the 1-bp deletion. The radiolabelled PCR products were electrophoresed on standard 6% polyacrylamide denaturing sequencing gels at 65 W for 2 h. The gels were then dried and autoradiographed. A representative example of an AGE analysis is shown in Fig. 1. All the cases exhibiting the 1-bp deletion were sequenced to confirm the 6174delT mutation. A small number of breast cancer cases not carrying the 6174delT mutation by AGE were included on the sequencing gel. Half of the cases were sequenced in one direction and half in the other direction, utilizing primer set 1 and primer set 2, respectively. Amplification was performed in 50 ul PCR reactions using the following forward and reverse primers: Set 1: TD-SFB: 5'–AATGATGAATGTAGCACGC–3' (*F* refers to forward primer); CGORF-RH: 5'–GTCTGAATGTTCGTTACT–3' (*R* refers to reverse primer). Set 2: BC11-RP and CGORF-RH. The products were electrophoresed on a 1% Nu-Sieve (FMC Corporation) gel, the bands excised from the gel, and the DNA fragments purified with Gene-Clean (Bio101). Cycle sequencing using the Cyclist DNA sequencing kit (Stratagene) was with primer CGORF-RH for the first set of seven samples and with BC11-RP for a second set of six samples. There was one sample in common between the two sets. The sequences were electrophoresed on 8% acrylamide/bis gels at 70 W for 2–3 h, the gels were dried and exposed to X-ray film. A representative sequencing reaction confirming the 6174delT mutation is shown in Fig. 2.

Acknowledgements

This work was supported by grants P20 CA 58232, RO1 CA65673, and RO1 CA55914 of the National Cancer Institute, grant DAMD17-94-J-H260 from the Department of Defense, and the Dewitt Wallace Fund. We thank R. Bogden and T. Davis of Myriad Genetics for supplying two of the sequencing primers, and L. Luzzatto for his critical reading of the manuscript.

Received 1 March; accepted 3 April 1995.

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Am. J. Hum. Genet. 58:271–280, 1996

Haplotype and Phenotype Analysis of Six Recurrent BRCA1 Mutations in 61 Families: Results of an International Study

Susan L. Neuhausen,[1,*] Sylvie Mazoyer,[4,*] Lori Friedman,[5,*] Michael Stratton,[6] Ken Offit,[8] Adelaide Caligo,[9] Gail Tomlinson,[10] Lisa Cannon-Albright,[2] Tim Bishop,[11] David Kelsell,[12] Ellen Solomon,[13] Barbara Weber,[14] Fergus Couch,[14] Jeffery Struewing,[15] Patricia Tonin,[16] Francine Durocher,[17] Steven Narod,[16] Mark H. Skolnick,[1,3] Gilbert Lenoir,[18] Olga Serova,[18] Bruce Ponder,[4] Dominique Stoppa-Lyonnet,[19] Douglas Easton,[7] Mary-Claire King,[5] and David E. Goldgar[1,†]

[1]Genetic Epidemiology Group, Department of Medical Informatics, and [2]Department of Internal Medicine, University of Utah School of Medicine, and [3]Myriad Genetics, Salt Lake City; [4]CRC Human Cancer Genetics Research Group, Department of Pathology, University of Cambridge, Cambridge; [5]Department of Molecular and Cell Biology and School of Public Health, University of California, Berkeley; [6]Section of Molecular Carcinogenesis and [7]Section of Epidemiology, Institute of Cancer Research, Sutton; [8]Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York; [9]Instituto Anatomia Patologiae, Pisa; [10]Department of Pediatrics, University of Texas Southwestern, Dallas; [11]Genetic Epidemiology Laboratory, Imperial Cancer Research Fund, Leeds; [12]Clare Hall Laboratories, Imperial Cancer Research Fund, and [13]Lincoln's Inn Fields, Imperial Cancer Research Fund, London; [14]Department of Hematology-Oncology, University of Pennsylvania, Philadelphia; [15]Genetic Epidemiology Branch, National Cancer Institute, and NIH, Bethesda; [16]Department of Medical Genetics, McGill University, Montreal; [17]Department of Molecular Endocrinology, CHUL Research Center and Laval University, Quebec City; [18]International Agency for Research on Cancer, Lyon; and [19]Unité de Genetique Oncologique, Institut Curie, Paris

Summary

Several BRCA1 mutations have now been found to occur in geographically diverse breast and ovarian cancer families. To investigate mutation origin and mutation-specific phenotypes due to BRCA1, we constructed a haplotype of nine polymorphic markers within or immediately flanking the BRCA1 locus in a set of 61 breast/ovarian cancer families selected for having one of six recurrent BRCA1 mutations. Tests of both mutations and family-specific differences in age at diagnosis were not significant. A comparison of the six mutations in the relative proportions of cases of breast and ovarian cancer was suggestive of an effect ($P = .069$), with 57% of women presumed affected because of the *1294 del 40* BRCA1 mutation having ovarian cancer, compared with 14% of affected women with the splice-site mutation in intron 5 of BRCA1. For the BRCA1 mutations studied here, the individual mutations are estimated to have arisen 9–170 generations ago. In general, a high degree of haplotype conservation across the region was observed, with haplotype differences most often due to mutations in the short-tandem-repeat markers, although some likely instances of recombination also were observed. For several of the instances, there was evidence for multiple, independent, BRCA1 mutational events.

Received September 26, 1995; accepted for publication November 15, 1995.

Address for correspondence and reprints: Dr. David Goldgar, Genetic Epidemiology, University of Utah School of Medicine, 391 Chipeta Way, Suite D2, Salt Lake City, UT 84108.

* These three authors contributed equally to this work.

† Present address: Unit of Genetic Epidemiology, International Agency for Research on Cancer, Lyon.

0002-9297/96/5802-0003$02.00

Introduction

The recent isolation of BRCA1, a gene predisposing to early-onset breast cancer and ovarian cancer (Miki et al. 1994), resulted in rapid identification of a large number of families with BRCA1 mutations (Castilla et al. 1994; Friedman et al. 1994; Hogervorst et al. 1995; Shattuck-Eidens et al. 1995). Although there were a large number of distinct mutations, several mutations were recurrent in a number of independently ascertained families of apparently diverse geographical origin, including two mutations, *5382 ins C* and *185 del AG*, each of which was identified in at least 10% of families in which the BRCA1 gene was completely sequenced (Shattuck-Eidens et al. 1995). A preliminary analysis of haplotypes of these two mutations among a set of Canadian families (Simard et al. 1994) showed that all four families with each mutation had (or could have) identical haplotypes at four short tandem repeat (STR) markers within, or within 50 kb of, BRCA1. With this observation, we examined haplotype conservation over a larger segment of DNA surrounding BRCA1 and in a larger, and, it is presumed, a geographically and ethnically more diverse set of families. In addition to the two mutations described above, several more mutations have been found in sufficient numbers (four or more times) to permit a preliminary examination of their associated haplotypes and phenotypes. The question arises whether these multiple instances of specific mutations represent independent mutational events or represent a founder effect many generations in the past. A related question involves inferences that can be made about the relative ages of individual BRCA1 mutations through analysis of haplotype conservation.

Genes responsible for inherited cancer, like many

other disease genes, have been associated with a wide diversity of expression. This is seen not only in variability in the age at diagnosis of cancer but also in the anatomical site in which the tumor originates. More important, at least from the clinical perspective, is the degree to which specific mutations and accompanying genetic backgrounds influence the expression of BRCA1 in terms of site and age at diagnosis. It has been recognized for several years that BRCA1 conveys a high risk of ovarian cancer in addition to early-onset breast cancer (Narod et al. 1991). However, in a recent analysis of a group of 17q-linked breast/ovarian families, Easton et al. (1995) found that while the overall risk of ovarian cancer was high (44% by age 70 years), there was statistically significant evidence for heterogeneity among families of this risk. It was estimated that while the majority (89%) of families had an elevated, but relatively modest, risk of ovarian cancer of .23, the remaining 10% of families had a markedly high lifetime risk of ovarian cancer of .85, similar to the risk of breast cancer in these families. These analyses were performed before BRCA1 was isolated and specific mutations could be categorized, and it was assumed that this heterogeneity of ovarian cancer risk would turn out to be due to specific mutations or mutations in particular domains of the coding sequence.

Studies of other diseases have shown that fine-structure haplotypic analysis can provide insights into the "genetic history" of a particular mutation (or presumed mutation for rare diseases where the disease gene is not yet identified) (Morral et al. 1993; Risch et al. 1995; Thomas et al. 1995). In the present paper, we address both the question of mutation origin and the relationship between mutation and phenotype. To do this, we constructed a haplotype of nine polymorphic STR markers within or immediately flanking the BRCA1 locus in a set of 61 families (selected to contain one of six BRCA1 mutations that had been identified a minimum of four times) and analyzed the phenotype associated with each mutation.

Methods

Genotyping of 17q Markers

Genotyping was carried out at three centers. The families collected by the University of California, Berkeley group (BERK) and the Cancer Research Campaign (CRC) centers in the UK were genotyped in Berkeley and in Cambridge, respectively. All other families were genotyped in the genetic epidemiology laboratory at the University of Utah. A similar protocol was followed at all centers. From 50 ml of ACD blood, nuclear pellets were extracted from white blood cells and DNA extracted with phenol and chloroform, precipitated with ethanol and resuspended in Tris-EDTA. All nine markers genotyped were STR loci (table 1) assayed by PCR by using standard procedures. Radiolabeled PCR products were electrophoresed on standard 6% polyacrylamide denaturing sequencing gels. Allele frequencies used in the likelihood calculations were as reported in the Genome Data Base (GDB) from typings of CEPH independent chromosomes (see table 1). Allele sizes were matched according to the genotype of CEPH reference individual 134702 who was used as a control on each gel.

BRCA1 mutations were identified in families included in this study by a variety of techniques, primarily direct sequencing or SSCP assay (Orita et al. 1989). Where possible, haplotypes associated with each mutation were inferred from multiple samples of related individuals within each kindred, known or presumed to have the same mutation; otherwise, multilocus genotypes were compared. The mutation in each family segregating a substantially different haplotype from the majority of families in the same mutation class was verified by allele-specific oligonucleotide hybridization.

Analysis of Haplotype Data

The estimation of the age of the mutations was performed using the following statistical model. The joint likelihood of the number of haplotypes (NHAP) BRCA1 haplotypes from families with a given mutation that is composed of alleles $\{a_m\}$ relative to the presumed ancestral haplotype $\{h_m\}$, $m = 1, \ldots$ NL (number of loci) STR markers, is given by

$$L = \prod_{i=1}^{\mathrm{NHAP}} \left[\mu_D \sum_{j=1}^{\mathrm{NC}_i} \prod_{m=1}^{\mathrm{NL}} p(a_{jm}) + (1-\mu_D) \sum_{j=1}^{\mathrm{NC}_i} L_j(1) \prod_{m=1}^{\mathrm{NL}-1} \frac{L_j(m; m+1)}{L_j(m)} \right]$$

where $L_j(m; n = m + 1) =$

$$(1-\mu_m)(1-\mu_n)\{\theta_m p(h_m) p(h_n) + (1-\theta_m)[1-\theta_{mn}+\theta_n p(h_n)]\} \quad \text{if } a_{jm} = h_m \text{ and } a_{jn} = h_n;$$

$$(1-\mu_m)\{\theta_m p(h_m)[\mu_n + (1-\mu_n)p(a_{jn})] + (1-\theta_m)[\mu_n + (1-\mu_n)\theta_{mn} p(a_{jn})]\} \quad \text{if } a_{jm} = h_m \text{ and } a_{jn} \neq h;$$

$$\mu_m(1-\mu_n)[\theta_n p(h_n) + 1 - \theta_n] + (1-\mu_m)\theta_m p(a_{jm})(1-\mu_n) p(h_n) \quad \text{if } a_{jm} \neq h_m \text{ and } a_{jn} = h_n;$$

Table 1

Allele Designations and Estimated Allele Frequencies of the Nine STR Markers Used for Haplotype Construction

	D17S . . .								
ALLELE	1185	1320	1321	855	1322	1323	1327	1326	1325
1	.01	.001	. . .	.01	.04	.02	.01	.01	.03
2	.02	.001	.03	.05	.03	.04	.01	.06	.02
3	.09	.04	. . .	.19	.11	.19	.01	.25	.04
4	.19	.09	.11	.18	.25	.03	.01	.08	.07
5	.19	.498	. . .	.15	.51	.04	.01	.28	.03
6	.17	.07	.02	.12	.04	.67	.03	.01	.02
7	.09	.27	. . .	.25	.01	.01	.03	.01	.04
8	.03	.03	. . .	.05	. . .	. . .	.08	.01	.10
9	.04	. . .	. . .	. . .	. . .	. . .	.05	.01	.12
10	.04	. . .	. . .	. . .	. . .	. . .	.05	.28	.19
11	.04	. . .	. . .	. . .	. . .	. . .	.08	. . .	.16
12	.09	. . .	.09	. . .	. . .	. . .	.63	. . .	.09
13	. . .	. . .	.01	. . .	. . .	. . .	.01	. . .	.09
14	. . .	. . .	.16	. . .	. . .	. . .	.005	. . .	. . .
16	. . .	. . .	.08	. . .	. . .	. . .	. . .	. . .	. . .
19	. . .	. . .	.01	. . .	. . .	. . .	. . .	. . .	. . .
20	. . .	. . .	.05	. . .	. . .	. . .	. . .	. . .	. . .
22	. . .	. . .	.07	. . .	. . .	. . .	. . .	. . .	. . .
23	. . .	. . .	.11	. . .	. . .	. . .	. . .	. . .	. . .
RG[a]	3,4	5,6	20,20	5,7	4,5	3,6	12,12	5,10	4,6

NOTE.—Only allele frequencies for alleles observed in our sample are given.

[a] RG—Reference genotype of CEPH individual 1347-02. The size of each allele can be found in the GDB on-line database.

$$\mu_m\{\mu_n + (1 - \mu_n)\theta_n p(a_{jn})\}$$
$$+ (1 - \mu_m)\theta_m p(a_m)\{\mu_n + (1 - \mu_n)p(a_{jn})\}$$
$$\text{if} \quad a_{jm} \neq h_m \quad \text{and} \quad a_{jn} \neq h_n ;$$

and $L_j(m) =$

$$(1 - \mu_m)[\theta_m p(h_m) + (1 - \theta_m)] \quad \text{if} \quad a_m = h_m$$
$$\mu_m + (1 - \mu_m)\theta_m p(a_{jm}) \quad \text{otherwise} ,$$

where the summation over j is over all possible number of combinations (NC) haplotypes for the mutation that are consistent with the observed genotypic data; μ_D is the probability of an independent, identical mutation at BRCA1. This was taken to be .0002 for the three mutations involving the insertion, deletion, or substitution of a single nucleotide; .0001 for the 2-bp deletion; .00005 for the 4-bp deletion; and .00001 for the 40-bp deletion; $\theta_m = 1 - \exp(-XG/1{,}000)$ is the probability of recombination between locus m at distance X kb from the BRCA1 mutation in G generations, if it is assumed that the genome average of 1 cM = 1 Mb and that the map in figure 1 is accurate; θ_{mn} is the similar quantity for recombination between locus m and locus n; $\mu_m = 1 - \exp(-G\mu^*)$ is the probability of a mutation at locus from allele h_m to allele a_m, $h_m \neq a_{jm}$, at locus m in G generations, where $\mu = .0006$ if locus m is a dinucleotide repeat, $\mu = .002$ if locus m is a tri- or tetranucleotide repeat (Weber and Wong 1993); $p(h_m)$ and $p(a_{jm})$ are the allele frequencies (table 1) of the presumed ancestral allele "h" at locus m on the presumed ancestral mutation-bearing haplotype and the corresponding allele frequency of the allele "a" observed in the jth possible derivative haplotype, respectively.

The method of maximum likelihood was used to find the value of G, the number of generations since the origin of the mutation, which best fit the pattern of haplotype sharing at the nine marker loci among families with identical mutations by using the equations given above. In this analysis it was assumed that each haplotype either arose from the ancestral haplotype or was the result of a second independent identical mutation. Starting with the location of the BRCA1 mutation, the loci were examined sequentially in both the proximal and distal directions; the likelihood of observing the marker allele at each locus was calculated conditional on the result at the preceding locus. Approximate support intervals for the age of each mutation were calculated by finding the value of G on either side of the most likely value which had at least a 10-fold decrease in likelihood. For each family with a mutation, the posterior probability that the corresponding haplotype arose through a new mutation at BRCA1 or through recombi-

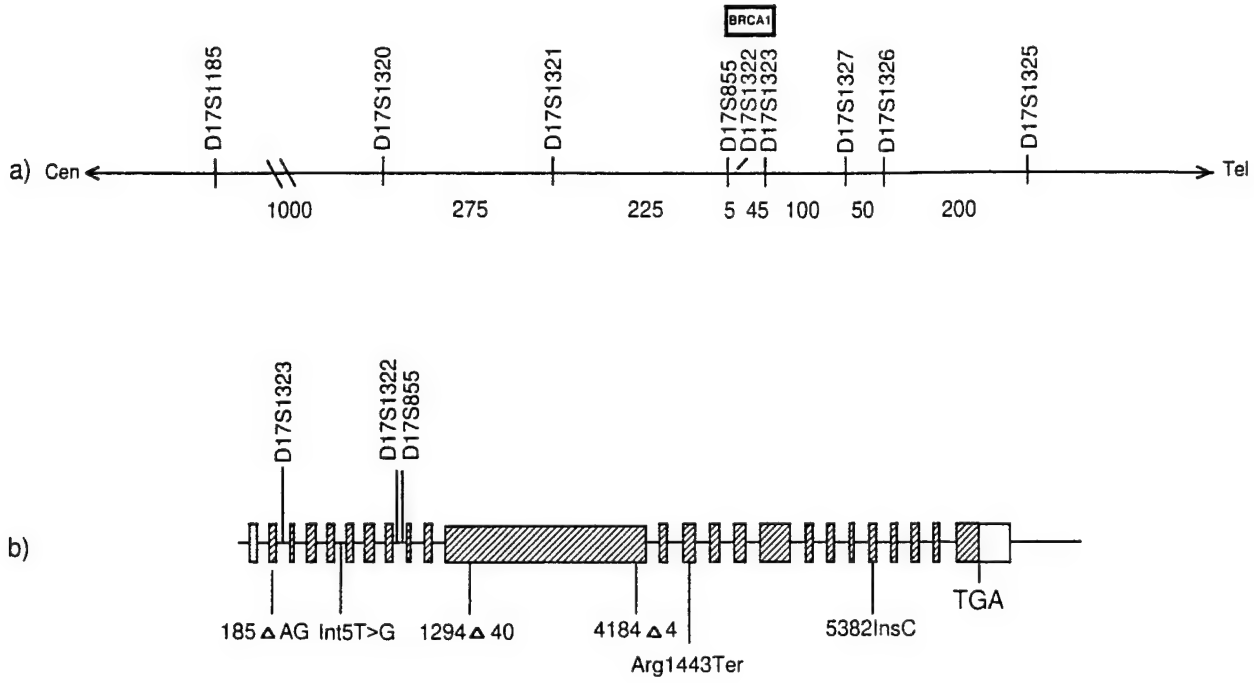

Figure 1 *a*, Physical map of the nine markers used in the analysis. The location of the BRCA1 locus with respect to these markers is shown. Distances (in kb) between markers are taken from Neuhausen et al. (1994), with the exception of D17S1185 which was placed on the map as part of the present study. *b*, Schematic diagram of the BRCA1 gene showing the position of the six mutations analyzed relative to the three intragenic markers.

nation and mutation at the marker loci from the presumed ancestral haplotype was calculated assuming a prior probability of μ_D relevant to that specific mutation. The sum across family haplotypes of these posterior probabilities yields an estimate for each mutation of the proportion of families derived from the presumed common ancestral haplotype. The computer software to implement this algorithm is available from one of the authors (D.E.G.).

Analysis of Phenotype Data

Phenotypic analysis was performed using the GLM and VARCOMP procedures of the SAS statistical analysis package (Cary). To test for heterogeneity in proportion of affected who had ovarian cancer among the six mutations, a randomization test was performed. Specifically, random permutations of families among the six mutations were performed, keeping the number of families with each mutation equal to that of the observed. Each such permutation resulted in a different six-by-two table with an associated χ^2 statistic calculated in the standard fashion. The χ^2 statistic associated with the observed aggregation of cases among mutations was compared with those calculated from 2,000 random permutations of the families among mutations. The rank of the observed χ^2 among those from 2,000 replicates is the nominal P value for testing the association of ovarian prevalence with specific mutation. The S-Plus package (StatSci) was used to perform the randomization test.

Results

Haplotype Analysis and Age of Mutations

The mutations described in this report encompass a wide variety of DNA sequence variants, including small insertions or deletions in both the 3′ and 5′ portions of BRCA1, a relatively large 40-bp deletion, a splice site mutation, and a nonsense mutation. The only relatively frequent BRCA1 mutation not represented in this study is the missense mutation *Cys 61 Gly* for which insufficient families were available for analysis when the study was initiated. Together, the six mutations analyzed account for approximately one-third of all mutations identified in >180 families in which BRCA1 mutations were found through screening the entire BRCA1 coding region, and they encompass the most frequently observed mutations to date.

The families that were genotyped are described in table 2 and are grouped by their respective mutations. In most instances there was clear haplotype sharing among independent families harboring the same BRCA1 mutation (table 2). The allele frequencies (table 1) and the genotypic data (table 2) were used with the statistical model described in Methods to estimate the relative age of each of the mutations. Likelihoods of the genotypic

data as a function of the number of generations since the original ancestral mutation were computed, and the value that maximized the likelihood was found through direct search. Approximate 90% confidence intervals were derived by finding bounds corresponding to a difference of 1.0 on the $\log_{10}$ likelihood scale. For each of the six mutations studied, the estimated number of generations G with the 1 LOD support intervals is shown in table 3. An estimate of the number of independent mutations required to best explain the observed data (table 3) is taken to be the sum of the posterior probabilities that each haplotype is the result of a new mutation relative to the likelihood that the haplotype is derived from the ancestral haplotype through mutation and/or recombination. For example, we estimate the *5382 ins C* to have occurred 38 generations ago, 90% limits (18,69), and that there is a 31% chance that one of the haplotypes arose from an independent identical mutation.

Among the 21 families in which the *5382 ins C* mutation was identified in one or more breast or ovarian cancer patients, all but two families were segregating the "4" allele at D17S855 and the "14" allele at D17S1327. The frequency of the "4" allele is .18, while the "14" allele is not present in 80 CEPH chromosomes and is rarely found outside the context of the accompanying mutation. The two exceptions are family ICR 82 and family MICH 205. The most probable explanation in both of these cases is a single mutation occurring at D17S1327 and D17S855, respectively. In the case of family 82, the haplotype was identical to the consensus haplotype with the exception of a "13" allele at D17S1327 instead of the typical "14" allele 2 bp smaller. This allele is rare in Caucasians, but, it is interesting, appears to be quite common in African Americans. While the majority of the families studied with this mutation primarily were of northern European descent, two samples were derived from families of eastern European origin, while another was identified in a family currently residing near Pisa, Italy.

The 2-bp deletion in codon 23, *185 del AG*, has been found predominantly in breast and ovarian cancer families of Ashkenazi Jewish ancestry (Struewing et al. 1995*b*; Tonin et al. 1995). Recently, this mutation has been found to have a frequency of 1% in the Ashkenazi Jewish population (Struewing et al. 1995*a*). Of the 19 families with this mutation analyzed in the present study, all but two are also of Ashkenazi Jewish descent. The two exceptions to this finding are the two families ascertained in Yorkshire, England, ICRF-BOV3 and ICRF-543, which are not (as far as can be determined) of Jewish ancestry and are not known to be immediately related to each other. These two families share a BRCA1 haplotype which bears little resemblance to that found in the other families with this mutation. Indeed, the statistical analysis provides overwhelming evidence that breast/ovarian cancer in both these families is due to an independent *185 del AG* mutation. The estimated age of the *185 del AG* mutation is 46 generations, or 920 years (assuming an average of 20 years per generation) with support limits of 460–1,600. Of the 17 families that likely derive from a common ancestral haplotype, three were segregating an allele 2 bp smaller for D17S1327 than in the other 14 families. If we assume that these represent the occurrence of a single mutation at this locus soon after the initial mutational event at BRCA1, rather than three independent mutations, as is assumed by the likelihood analysis, then the estimated age is reduced to 38 generations, or 760 years. Thus, under the assumed model, the most likely date for the origin of the 185 del AG mutation found in the Ashkenazi Jewish population is ~1235 A.D.

Inspection of the haplotypes found in families with the *4184 del 4* mutation reveals considerable apparent diversity in these haplotypes. Perhaps, not coincidentally, the *4184 del 4* mutation was the only mutation in which non-Caucasian samples were represented; there were two families, ICR227 and CRC OV44 currently living in England but who were recent immigrants from Pakistan and India, respectively. According to the likelihood analysis, breast/ovarian cancer in both of these families was the result of an independent mutation. A third family, BERK 2, also appears to have a different haplotype than the three families that appear to share a common haplotype; it is interesting that this family is of Norwegian ancestry. Without considering the prior probability of an identical 4-bp deletion, it is ~1,500 times more likely that the mutation in this family was the result of an independent mutation. However, in light of the prior probability of 1/20,000 assigned to this mutation, the posterior probability that this is in fact a new mutation is only .07. It is more likely that this haplotype arose as a result of a recombination event within the BRCA1 gene, thus contributing to the relatively large estimated age of the *4184 del 4* mutation; it should be noted, however, that because of the limited amount of data, the support interval for the age of this mutation is very large.

For the sole nonsense mutation studied, *Arg 1443 ter*, there were also at least three independent mutations, as deduced by both inspection of haplotypes and the likelihood analysis. Because the other two families with this mutation could have identical haplotypes, no information was available to estimate the age of the putative ancestral mutation, resulting in a maximum-likelihood estimate at the boundary of a single generation.

Association of Phenotypic Variation with Common Mutations

A summary of the number of cases of breast and ovarian cancer and the ages at diagnosis of the breast cancer

Table 2

Haplotype and Phenotype Data for the 61 Families Studied

Mutation and Center (family)	No. of Cases[a]										
	Breast	Ovarian	D17S1185	D17S1320	D17S1321	D17S855	D17S1322	D17S1323	D17S1327	D17S1326	D17S1325
5382 ins C:											
UTAH (1910)	4	0	5	7	20	4	5	6	14	4	10
UTAH (2339)	6	4	5	7	14	4	5	6	14	4	10
UTAH (2368)	3	2	5	7	20	4	5	6	14	4	10
MONT (101)	1	2	5/8	7	20	4	5	6	14	4	10
MONT (279)	3	1	5	7	20	4	5	6	14	4	10
MONT (162)	2	1	4/5	7	20/14	4	5	6	14/12	3/4	6/10
MONT (166)	3	2	...	...	...	4/7	3/5	3/6	14/7	...	...
CURIE (342)	2	2	7	7	14	4	5	6	14	4	10
CURIE (49)	4	0	5/7	7	20	4	5	6	14/12	4	10
CURIE (417)	4	0	5	5/7	20/21	4/5	3/5	6	14/10	4/10	1/10
CURIE (360)	2	1	5	7	16/22	4/7	3/5	3/6	14/4	4/10	10/11
CURIE (461)	1	1	5/6	...	16	4	5	6/7	14/12	4/5	10
CURIE (508)	4	0	5/6	4/7	16/20	4/5	5	6	14/12	4/5	10/2
NCI (3365)	2	1	3/4	5/7	20/23	4/7	3/5	1/6	14/5	4/10	10/8
ICR (82)	4	0	5/7	7	...	4	5	6	13	4	10
CRC (999)	0	1	2/5	4/7	20/25	4/8	...	...	14/10	4/10	10/7
PISA (999)	1	3	...	3/7	14/16	4	5	6	14/12	4/2	10
BERK (95)	5	0	4	7	20	4	5	6	14	4	10
MICH (205)	6	1	5	...	19/22	5	5/4	6	14	4	10
MSK (25)	0	3	...	7	...	4	5	6	14/12	4	...
IARC (1973)	5	1	...	...	...	4	5	6	14	4	...
185 del AG:											
MONT (235)	4	2	3	7/8	14	7	3	3	6	10	6
MONT (255)	5	7	3/5	7/8	14/16	7	3	3	6	10	4/6
MONT (253)	1	3	...	...	...	7/8	3/4	2/3	6/8	...	...
MONT (180)	2	2	...	...	...	7/8	3/5	3/6	6/11	...	...
MSK (43)	3	0	3	7	14	7	3/5	3	7	10	5
NCI (167)	8	2	5	7	14	7	3	...	7	10	13
NCI (3279)	0	3	4	5/7	14	4/7	3/5	3/6	6/12	3/10	5/9
NCI (62)	6	3	3/4	5/7	14/20	7	3/5	3	4/6	10	5/13
ICRF (BOV3)	12	2	6	7	14	4	5	7	12	5/10	10
ICRF (543)	3	0	...	7	14	4	5	7	12	5	10

BERK (B28)	2	0	4	7	14	7	3	3	6	10	5
BERK (B8)	4	0	3	7	14	7	3	3	7	10	5
BERK (117)	3	0	3	7	14	7	3	3	6	10	5
BERK (139)	2	2	4	7	14	4/7	3	3/5	6/12	10	5/10
BERK (144)	2	2	3	7	14	7	3	3	6	10	5
CURIE (72)	4	0	3/4	5/7	13/19	7	3/5	3	4/6	10	5/13
CRC (OV10)	1	3	3	7	14	7	3	3	6	3	7/10
IARC (2979)	2	3	...	...	...	7	3	3	6	10	...
IARC (3079)	2	4	...	...	...	7	3	3	6	10	...
4184 del 4:											
MONT (183)	4	1	3	7/8	4	1	6	6	12	3	10
ICR (227)	3	1	4/6	3/7	12	5	5	6	12	3	10
ICR (229)	2	2	4/9	1	4	1	6	6	12	3/10	13
CRC (OV44)	0	3	3	7	22	8	1	3	7	10	13
BERK (B10)	8	0	4	7	4	1	6	6	12	3	11
BERK (2)	4	1	5	8	4	3	5	6	12	5	10
1294 del 40:											
MONT (185)	1	3	8	3	14/16	6	5	6	12	3	10
ICR (B138)	5	1	7/8	3	14/16	6	5	6	12	3/10	10
MICH (32)	5	1	8	3	14/16	6	5	6	12	3	10
CRC (OV2)	1	4	8	3	14	6	5	6	12	10	5
CRC (OV150)	0	4	8	3/7	4/14	6/8	5	6	12	5	8/10
NCI (1138)	1	4	7/8	3/5	14/22	3/6	...	...	12	3	...
Intron 5 T→G ins 59 ter 75:											
UTAH (1001)	12	0	7	5	23	5	5	6	12	5	9
UTAH (2301)	11	3	7	5	23	5	5	6	12	5	10
BERK (82)	4	2	6	5	23	5	5	6	12	5	6
UTSW (211)	3	1	5/6	5	23	5	5	6	12	5	7
IARC (2749)	6	0	...	...	...	5	5	6	12	5	...
Arg 1443 ter:											
STR (1900)	3	1	5/6	5/7	20/22	5	5	6	12	3/5	7/10
ICR (B35)	4	0	3/4	5/7	14	7	4	2	7	10	6
ICR (B155)	4	2	6	3/5	...	5/8	...	6	12	5	1/13
BERK (81)	10	1	6	7	22	5 & 6	5	6	12	3	7

NOTE.—In cases where the allele segregating with BRCA1 was impossible to determine from available data, the genotype of the proband in whom the mutation was identified is given. Only breast cancer cases diagnosed under the age of 65 are included. In families with linkage and/or mutation data, cases of breast and ovarian cancer which do not share the linked haplotype/mutation have been excluded; cases of breast or ovarian cancer of unknown status are presumed to be due to BRCA1.

[a] Women with both breast and ovarian cancer are counted in both the breast and ovarian columns.

Table 3

Estimated Number of Generations (*G*) (with One-LOD Support Intervals) for Each Mutation

Mutation	*G*	One-LOD Support Interval	Proportion of Families[a]
185 Del AG	46	(23, 80)	.89
Intron 5 Splice	75	(19, 165)	1.00
1294 Del 40	9	(1, 45)	.84
4184 Del 4	170	(70, 350)	.66
Arg 1443 Ter	1	(1, 63)	.50
5382 Ins C	38	(18, 68)	.99

[a] Estimated proportion of families within each mutation class with cancer due to the mutation occuring on the presumed ancestral haplotype.

cases stratified by BRCA1 mutation type is shown in table 4. There was little apparent variation in age at diagnosis as a function of mutation, although the two mutations with the lowest fraction of ovarian cancer cases had lower median age at diagnosis of breast cancer. The ages of the breast cancer cases among the families with the mutations associated with the highest rates of ovarian cancer (*1294 del 40* and *185 del AG*), conversely, tended to have slightly higher median age at diagnosis. However, overall, little of the variation observed in age at diagnosis among the 219 cases of breast cancer could be apportioned to mutation or family-specific sources (estimates of 2% and 0%, respectively). A test of mutation-specific differences in age at diagnosis computed using the expected mean squares from an unbalanced nested analysis of variance was not significant ($F_{5,49} = 1.58$, $P = .18$). The randomization test described in Methods was used to examine possible differences in the relative proportions of cases of breast and ovarian cancer among the six mutations. The observed χ^2 of 19.04 was 138th largest among 2,000 random permutations of the families among mutation class, corresponding to a P value of .069.

Discussion

Previous studies have used analysis of markers linked to a disease locus to more precisely localize an unknown disease gene with respect to those markers (Hästbacka et al 1992; Ramsay et al 1993; Kaplan et al. 1995). In those situations, the population is often a geographic/genetic isolate derived from a relatively small set of founders; it is generally assumed that the age of the mutation is known; and it is the location of the unknown disease locus with respect to the marker loci that is to be estimated. Here we have examined a known disease gene in conjunction with a set of markers whose physical distances from the disease gene are known and use data from similarities and differences of the marker haplotypes in families with identical mutations to make inferences about the number of independent identical ancestral mutations, the age of each of these mutations, and the mutation rates at the marker loci.

In the case of the BRCA1 mutations studied here, the individual mutations are estimated to have arisen 180–3,400 years ago. For comparison, a similar study of haplotype conservation in >1,000 cystic fibrosis–bearing chromosomes estimated that the common Δ508 mutation arose at least 53,000 years ago, while two other mutations were believed to have originated ~35,000 years ago (Morral et al. 1993). In light of the relatively small amount of data present in our study, even for the two most common mutations, the results of the likelihood analysis of mutation age are very sensitive to the assumptions about the model, particularly the assumptions of a uniform relationship between recombination rate and physical distance and equal mutation rates

Table 4

Breast and Ovarian Cancer Phenotypes Associated with Each of the Six Mutations Studied

		Breast Cancer		Ovarian Cancer	
Mutation	No. of Families	No. of Cases	Median Age at Diagnosis[a]	No. of Cases	% OV[b]
185 Del AG	19	66	42	38	37
Intron 5 Splice	5	36	40	6	14
1294 Del 40	6	13	42	17	57
4184 Del 4	6	21	39	8	28
Arg 1443 Ter	4	21	36	4	16
5382 Ins C	21	62	41	26	30
Total	61	219	41	99	31

[a] Median age at diagnosis of breast cancer among the cases associated with each mutation.
[b] Proportion of total breast and ovarian cancer cases associated with each mutation that were ovarian.

among all STRs of the same class. In particular, the results of the analysis appear to be quite sensitive to these marker mutation rates. To examine this effect, we reanalyzed the data for the two most common mutations, *5382 ins C* and *185 del AG*, assuming STR marker mutation rates were an order of magnitude (i.e., 10×) lower and higher than the values used in the original analysis. For *5382 ins C*, the estimated age of this mutation was only 9 generations (in contrast to the estimated age of 38 generations given in table 3) when a 10-fold increase in STR mutation rates was assumed and 50 generations if mutations at the marker loci occurred at one-tenth the assumed frequency of 6×10^{-4} (Weber and Wong 1993). Similarly, under these values of marker mutation rates, the maximum-likelihood estimates of the age of the *185 del AG* mutation are 11 and 65 generations, as compared to the value of 46 generations estimated using the frequencies reported in Weber and Wong (1993). However, even the limited information available for each mutation at this early stage of data collection should provide some clues to the genetic history of each of these mutations. Moreover, the model and computer implementation described here are the most complex of their kind for multipoint haplotype data and will be useful in the analysis both of BRCA1 mutations as more data become available, and of other disease-causing mutations for which such data may already exist.

Risch et al. (1995), in a study of haplotypes surrounding the locus for idiopathic torsion dystonia (IDT) in 59 Ashkenazi Jewish families, estimated the age of the single, founder, disease-causing mutation to be 350 years, although that study assumed a 25-year generation, while we assumed an average generation of 20 years. It should be noted that although the origin of the IDT mutation appears much earlier than the *185 del AG* mutation in the same population, the support intervals overlap and differences in the models used make comparisons between studies difficult.

The second objective of this study was to partition the phenotypic variance observed in BRCA1 to family- and mutation-specific components. Two phenotypes were available for this analysis: age at diagnosis of each breast cancer case and the relative numbers of breast and ovarian cancer in each family. Very little of the variation in age at diagnosis of breast cancer was due to mutation-specific effects, and the variation within families was greater than that observed between families, indicating the lack of specific familial factors involved in predicting age at diagnosis of breast cancer in these BRCA1 families. It is likely that much of the variation in age at onset of breast cancer in BRCA1 carriers is simply the result of stochastic variation predicted by the two-hit model of carcinogenesis for tumor-suppressor genes first proposed by Knudson (1971). There was more evidence for mutation-specific variation in the relative proportion of ovarian cancers, with values ranging from 57% of affected women having ovarian cancer for the *1294 del 40* mutation, to only 14% for the intron-5 splice mutation. A randomization test for mutational differences in the relative number of breast and ovarian cancers was suggestive of an effect ($P = .07$), but not definitive. However, in light of the available data, the analysis did not take into account the pedigree structure, the number of women at risk, or censoring due to oophorectomy. As these data become available, and as mutation-carrier status is determined in both affected and unaffected women in these families, a more complete analysis, comparing age-specific risks of both breast and ovarian cancer can be undertaken.

In a preliminary study of this type, using data from four extended Utah kindreds (two of which are included in this report, K2301 and K1001), Goldgar et al. (in press) found significant differences in risk among three mutations studied. One hypothesis that has been put forward to explain heterogeneity of risk among families and/or mutations has been the length of the truncated protein product (Shattuck-Eidens et al. 1995). In the present study we did not examine location of the mutation as a specific factor, but it is interesting that the splice-site mutation with the apparent low prevalence of ovarian cancer results in a predicted truncated protein at codon 75 of BRCA1, while the mutation *1294 del 40*, which by contrast has a quite high ovarian cancer prevalence, has a predicted length of 397 amino acids. It is also possible that observed family-specific variation in clinical expression of BRCA1 (Easton et al. 1995) reflects the effect of linked modifier loci or familial clustering of environmental factors interacting with the BRCA1 genotype.

Reproductive risk factors and potential modifiers genes that have been associated with breast and ovarian cancer risks in the general population through case-control studies should now be examined in families with BRCA1 mutations to assess their contribution to this variation. These studies will require a larger sample size and complete pedigrees with detailed information on each individual who carries an altered BRCA1-susceptibility allele.

Acknowledgments

The authors wish to thank the many families who participated in the research studies described in this study. We also gratefully acknowledge the efforts of S. Gayther at Cambridge University, Bill Warren at the Institute of Cancer Research, and Donna Shattuck-Eidens at Myriad Genetics for identifying the unpublished mutations in the families that appear in this report. This work was supported by NIH grant CA-55914 to M.H.S., DOD grant DAMD 17-94-J-4260 to D.E.G., and

DOD grant DAMD 17-94-J-4299 to S.L.N. and was also supported by DOD grant 17-J-4299 and the Ontario Chapter of the Canadian Breast Cancer Foundation.

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The carrier frequency of the *BRCA2* 6174delT mutation among Ashkenazi Jewish individuals is approximately 1%

Carole Oddoux[1], Jeffery P. Struewing[2], C. Mark Clayton[1], Susan Neuhausen[3], Lawrence C. Brody[4], Michael Kaback[5], Bruce Haas[5], Larry Norton[7], Patrick Borgen[8], Suresh Jhanwar[6], David Goldgar[9], Harry Ostrer[1] & Kenneth Offit[6]

[1]*Human Genetics Program, Department of Pediatrics, New York University Medical Center, New York, New York 10016, USA*
[2]*Genetic Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA*
[3]*Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah 84108, USA*
[4]*Laboratory of Gene Transfer, National Center for Human Genome Research, National Institutes of Health, Bethesda, Maryland 20892, USA*
[5]*Department of Pediatrics and Reproductive Medicine, University of California, San Diego, San Diego, California 92123, USA*
[6]*Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA*
[7]*Breast Cancer Medicine Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA*
[8]*Breast Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York 10021*
[9]*Unit of Genetic Epidemiology, International Agency for Research on Cancer, Lyon, Cedex 08, France*

Correspondence should be addressed to H.O.
e-mail: ostreh01@mcvax.med.nyu.edu

Certain germline mutations in either *BRCA1* or *BRCA2* confer a lifetime risk of developing breast cancer that may approach 90%[1–3]. The *BRCA1* 185delAG mutation was found in 20% and the *BRCA2* 6174delT mutation in 8% of Ashkenazi Jewish women with early-onset breast cancer[1–6]. The 185delAG mutation was observed in 0.9% of 858 Ashkenazi Jews unselected for a personal or family history of cancer[7]. Assuming comparable age-specific penetrances, a carrier frequency of 0.3% was estimated for the 6174delT *BRCA2* mutation[6]. To test this hypothesis, we performed a population survey of 1,255 Jewish individuals. In two independent groups, a prevalence of approximately 1% (C.I. 0.6–1.5) was observed for the 6174delT mutation. The relative risk of developing breast cancer by age 42 was estimated to be 9.3 (C.I. 2.5–22.5) for 6174delT, compared to 31 (C.I. 11–77) for 185delAG. Analysis of 107 Ashkenazi Jewish women with breast cancer and a family history of breast or ovarian cancer confirmed a four-fold greater prevalence for the *BRCA1* 185delAG mutation compared to the *BRCA2* 6174delT mutation. Our findings suggest a difference in cumulative lifetime penetrance for the two mutations. Genetic counseling for the one in 50 Ashkenazi Jewish individuals harbouring specific germline mutations in *BRCA1* or *BRCA2* must be tailored to reflect the different risks associated with the two mutations.

Two genes, *BRCA1* and *BRCA2*, have been estimated to account for 60–80% of heritable predisposition to breast cancer[1–3,8,9]. The presence of a germline mutation in one of these genes increases the likelihood that breast and ovarian cancer will develop at an earlier age. Mutations in the *BRCA2* gene also increase risk for breast cancer in male heterozygotes[2]. A wide variety of germline mutations in *BRCA1* and *BRCA2* have been identified among high-risk families studied so far. By contrast, among Ashkenazi Jews identified on the basis of linkage studies or early-onset breast cancer, a number of common mutations have been found. For *BRCA1*, two frameshift mutations in exon 2, 185delAG and 188del11, and the 5382insC mutation in exon 20 are over-represented in families of Ashkenazi Jewish origin[10–14]. The 185delAG mutation is observed among 20% of women with early-onset breast cancer[1,5] and the carrier frequency of this mutation among Ashkenazi Jewish individuals unselected for personal or family history of breast cancer is reported to be 0.9% (C.I. 0.4–1.8)[7].

A frameshift mutation in *BRCA2*, 6174delT, was observed in 8% of 80 Ashkenazi Jewish women with early-onset breast cancer (before age 42)[6]. Assuming that the penetrance by age 42 is similar to the *BRCA1* 185delAG mutation, a carrier frequency of 0.3% was estimated. To determine the validity of this estimate, we have undertaken a population-based study of individuals unselected for personal or family history of cancer.

Two groups of individuals who presented for heterozygote detection of Tay-Sachs and other autosomal recessive conditions were tested anonymously (Table 1). Of 1255 individuals tested, the 6174delT mutation was observed in 12 (0.9%) [C.I. 0.5–1.6%]. No 6174delT mutation was observed in 519 non-Jewish individuals.

Because previous estimates of the prevalence of the *BRCA1* 185delAG and *BRCA2* 6174delT mutations were performed on early-onset cases, we analysed 29 probands with breast cancer diagnosed between the ages 42 and 80 years. Each proband had a family history of breast or ovarian cancer. We detected four *BRCA1* 185delAG mutations and one *BRCA2* 6174delT mutation. Combining these cases with those studied previously[5,6], a group of 107 Ashkenazi Jewish women with breast cancer and a family history of breast or ovarian cancer were analysed. Cases ranged from 20 to 80 years of age. There was a four-fold greater prevalence of the *BRCA1* 185delAG mutation compared to the *BRCA2* 6174delT mutation. Overall, 7 women had 6174 delT mutations compared to 28 women with the 185delAG mutations ($P < 0.001$). When cases were grouped according to age $<$ or $\geq$ 42 years, the proportion of heterozygotes in each group was the same for both mutations. Twelve (43%) of the 28 women with *BRCA1* 185delAG mutations were affected after age 42, compared to 3 (43%) of 7 women with *BRCA2* 6174 delT mutations ($P = 1$). The median age at diagnosis of the 185delAG cases was 41 years as compared to 45 years for the 6174delT cases ($P = 0.3$).

Based on our results screening 1,255 individuals, the prevalence of the *BRCA2* 6174delT mutation in the Ashkenazi Jewish population is comparable to that reported for the 185delAG mutation (0.9%)[7]. This is surprising in light of the differences in the frequencies of the *BRCA1* 185delAG and *BRCA2* 6174delT mutations observed among Ashkenazi Jewish women with breast cancer.

There are at least three possible explanations for the observed difference. First, the risks conferred by these

Table 1 Prevalence of *BRCA2* 6174delT mutation in Ashkenazi Jewish individuals and controls

Population	Source	Subjects tested	Subjects with 6174delT	Percent 6174delT (C.I.)
Ashkenazi Jewish	NYU Medical Ctr.	848	8	0.9
	NIH	407	4	0.9
	Total	1255	12	0.9 (0.6–1.5)
Non-Jewish	NYU Medical Ctr.	175	0	0 (0–0.5)
	NIH	344	0	0 (0–0.5)
	Total	519	0	0 (0–0.4)

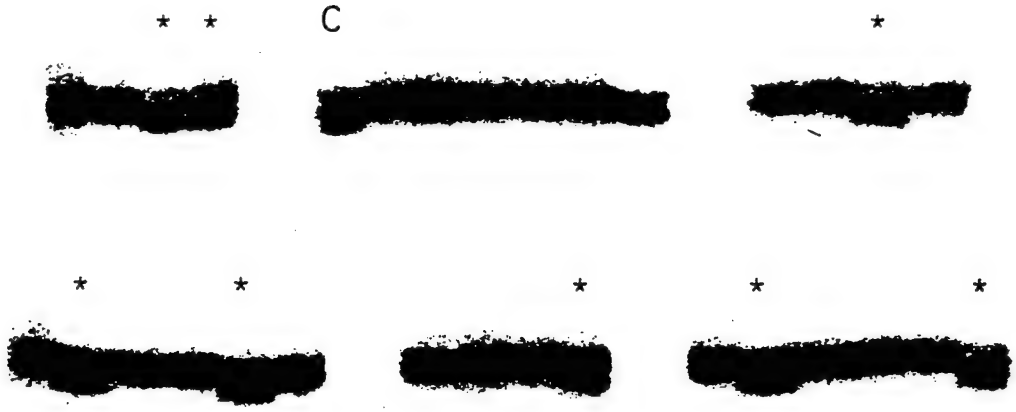

Fig. 1 Autoradiogram of positive (*), negative (no mark) and control (C) samples analysed by acrylamide gel electrophoresis of the PCR products. Positive samples include two main bands and a shadow band, whereas negative samples include one main band and a shadow band. The control sample was previously reported[6].

two mutations may in fact be equal, and the observed differences may reflect chance sampling variation among the sets of individuals with early-onset breast cancer. This possibility seems less likely since the prevalence of the two mutations was significantly different in the cohort of 107 Jewish breast cancer patients analysed. Second, the carrier frequency of the 6174delT mutation may be somewhat lower than that of the 185delAG, yet sampling variation yielded similar estimates for the two mutations. This seems unlikely since similar estimates of 6174delT were found independently in two sample sets of Ashkenazi Jewish individuals unselected for personal or family history of cancer (Table 1). One of the sample sets was a subset of a larger population utilised to estimate the prevalence estimate of 185delAG (ref. 7). In addition, the probability that the true prevalences are sufficiently different to account for the observed difference in the prevalence of the early-onset cases is quite small.

The third, and potentially most interesting, explanation for these results is that the *BRCA2* 6174delT mutation confers a different risk for breast cancer than does the *BRCA1* 185delAG mutation. Based on the frequency of the mutation in early-onset cases unselected for family history[6], and the carrier frequency reported here, the estimated relative risk of breast cancer by age 42 is 9.3 (C.I. 2.5–22.6) for the *BRCA2* 6174delT mutation, compared to 31 (C.I. 11–77) for the *BRCA1* 185delAG mutation. The four-fold lower prevalence of the *BRCA2* 6174delT mutation compared to the *BRCA1* 185delAG mutation suggests a difference in lifetime cumulative penetrance. This observation is supported by the lower prevalence of the 6174delT mutation also detected in the later-onset cases. In addition, a family history of breast or ovarian cancer was observed in all 16 185delAG heterozygotes in a prior series[3], but was absent in 2 6174delT heterozygotes[6].

Our findings suggest that the *BRCA2* 6174delT mutation has a different lifetime cumulative penetrance compared to the *BRCA1* 185delAG mutation. More precise estimates of the age-specific risks conferred by the *BRCA2* 6174delT mutation require additional case series unselected for personal or family history of cancer and long-term follow-up of large numbers of individuals who have this mutation. The observation that 1 in 50 Ashkenazi Jewish individuals may carry specific germline mutations of *BRCA1* or *BRCA2* will provide the opportunity to analyse the efficacy of medical and surgical interventions in this population. Genetic counseling for risk of breast and other cancers in unaffected relatives will need to be tailored to the specific mutation in each kindred.

Methods

Subjects. The subjects included individuals who presented for heterozygote detection of Tay-Sachs and Gaucher diseases and/or cystic fibrosis, as described[7,15]. At NYU Medical Center, this included 848 Ashkenazi Jewish individuals and 175 individuals who were not of Jewish origin. No information was obtained from these subjects about personal medical or family history. Prior to testing, personal identifiers were removed from the samples. At NIH, the subjects included 407 Ashkenazi Jewish individuals and 344 individuals who were not of Jewish origin[7]. This aspect of the study had the approval of the NYU Medical Center Institutional Board of Research Associates and the NIH Office of Human Subjects Review.

A second cohort consisted of 107 Ashkenazi Jewish patients at Memorial Sloan-Kettering Cancer Center with breast cancer and a family history of breast or ovarian cancer in a first or second degree relative. This was comprised of 29 previously unpublished cases in which the proband was diagnosed with breast cancer after age 42 in the setting of a family history of breast or ovarian cancer. These data were combined with those of 78 patients with a family history of breast or ovarian cancer included in a series of 107 breast cancer cases previously reported[6]. For this aspect of the study, informed consent was obtained from each patient, with results of genetic testing made available in the context of genetic counseling.

Mutation detection. DNA was prepared using Puregene reagents (Gentra Systems). For subjects with breast cancer, the *BRCA2* 6174delT mutation was detected by acrylamide gel electrophoresis as described[6]. For screening of the Tay-Sachs cohorts, at NYU this analysis was performed with the following modifications. PCR was performed in a microtiter format as 25 µl reactions. One of the PCR primers was end-P with α-^{32}P (Fig. 1)[16]. At the NIH, DNA was prepared as described[7]. A 3-primer PCR assay was designed using the TD-SFB, CGORF-RH, and a mutant-specific primer (6174delT-F, 5'-GGA TTT TTA GCA CAG CAA GG-3'). 50–100 ng genom-

ic DNA was amplified in a 20 µl reaction using 1× Perkin Elmer PCR buffer, 100 µM each dNTP, 100 nM TD-SFB, 200 nM each CGORF-RH & 6174delT-F, 2.5 mM $MgCl_2$, 20% sucrose, 100 µM cresol red, and 1 U *Taq* polymerase. Thermal cycling included a 2 min denaturation step at 94 °C, 35 cycles of 15 s at 94 °C, 15 s at 55 °C, 30 s at 72 °C, and a final extension step for 10 min at 72 °C. PCR products were separated on 1.8% agarose gels. This assay resulted in a single, 342-bp fragment in homozygous wild-type samples, and in the heterozygote, a wild-type 342-bp and a 143-bp fragment specific for the 6174delT mutation.

Mutations were confirmed by sequence analysis of PCR products. These products were generated using the TD-SFB and GCORF-RH primers and sequencing was performed using the GCORF-RH primer or the CGORF-RH and TD-SFB primers[6]. For sequencing at NYU Medical Center, 5 µl of the PCR product was treated with 2 U of shrimp alkaline phosphatase and 10 U exonuclease I (Amersham) at 37 °C for 15 min. After heat inactivation at 80 °C, 5 µl was cycle-sequenced using the Thermosequenase Kit (Amersham). The 6174delT mutation was confirmed in all eight mutant samples at NYU Medical Center and because of an insufficient quantity of DNA, three of the four mutant samples at NIH.

Estimate of the relative risk (R) of developing breast cancer before age 42 due to the 6174delT mutation. This was derived by utilising the equation $O=Rq/(Rq+1-q)$, where O is the proportion of breast cancer attributable to the 6174delT mutation and q is the carrier frequency of the mutation[6]. A simulation-based technique accounting for the sampling distribution inherent in the observed data provided an estimate of the distribution of mutation-specific risks for 6174delT. This was performed by assuming a disease-specific prevalence of 6/80 cases for early-onset breast cancer and a population prevalence of 12/1255 for 6174delT. Analysis of 10,000 replicates was performed to estimate the mean value for relative risks and confidence intervals, assuming the number of positives followed a Poisson distribution.

Acknowledgements

We thank L. Luzzatto for critical reading of the manuscript and J. Phillips for technical assistance. This work was supported by grants P20 CA 58232, R01 CA 65673, and R01 CA 55914 of the National Cancer Institute, grant DAMD17-94-J-4260 from the Department of Defense, the Dewitt Wallace Fund, and the National Foundation for Jewish Genetic Diseases.

Received 7 June; accepted 30 August 1996.

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Article

Germline *BRCA1* 185delAG mutations in Jewish women with breast cancer

Kenneth Offit, Teresa Gilewski, Peter McGuire, Alice Schluger, Heather Hampel, Karen Brown, Jeff Swensen, Susan Neuhausen, Mark Skolnick, Larry Norton, David Goldgar

Summary

Background We aimed to find out the proportion of breast cancers in Ashkenazi Jewish women attributable to the frameshift mutation at position 185 involving the deletion of adenine and guanine (185delAG) in the breast cancer gene *BRCA1*.

Methods We studied 107 Ashkenazi Jewish women with breast cancer seen at medical oncology and genetic counselling clinics in New York over a three and a half year period beginning in 1992. 80 of the women were diagnosed before age 42 years; the other 27 were diagnosed between 42 and 50 years and had a positive family history. Genomic DNA testing by PCR amplification was done to identify any 185delAG mutations of the *BRCA1* gene.

Findings Of the 80 women diagnosed before the age of 42 years, 16 (20%, 95% CI 11·2–28·8) were heterozygous for the mutation. All 16 women had at least one first-degree or second-degree relative with breast or ovarian cancer. Of 27 probands diagnosed with breast cancer between the ages of 42 and 50 years who had at least one first-degree relative affected with breast or ovarian cancer, 8 (30%, 95% CI 12–47) had 185delAG mutations.

Interpretation These data suggest that screening for the 185delAG mutation may be useful in genetic counselling of these women where options for detection and prevention of possible cancers can be discussed.

Lancet 1996; **347:** 1643–45

See Commentary page 1638

Clinical Genetics Service, Department of Human Genetics (K Offit MD, P McGuire BA, A Schluger MSc, H Hampel MSc, K Brown MSc) **and Breast Oncology Service, Department of Medicine** (T Gilewski MD, L Norton MD), **Memorial Sloan Kettering Cancer Center, New York, NY, USA; Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah, USA** (J Swensen PhD, S Neuhausen PhD, M Skolnick PhD, D Goldgar PhD); **Myriad Genetics Inc, Salt Lake City, Utah, USA** (M Skolnick); **and Unit of Genetic Epidemiology, International Agency for Research on Cancer, Lyon, France** (D Goldgar)

Correspondence to: Dr Kenneth Offit, Clinical Genetics Service, Department of Human Genetics, Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA

Introduction

Inherited mutations of the breast cancer 1 gene, *BRCA1*, may account for between a third and half of hereditary breast cancer, and for the majority of families with hereditary breast or ovarian cancer syndrome.[1,2] For women with inherited mutations of *BRCA1*, the lifetime risk of breast cancer may be as high as 90%. There is also an increased risk of ovarian cancer, which is not as clearly defined. A 2 base-pair (bp), adenine and guanine, deletion at position 185 in codon 23 of exon 2 (185delAG) is one of the commonest mutations of *BRCA1* described so far.[2] Subsequent reports[3–5] documented the presence of the 185delAG mutation in several Ashkenazi Jewish families with members who had breast and ovarian cancer.[3–5] 8 (0·9%) of 858 individuals of Ashkenazi Jewish ancestry seeking genetic screening for cystic fibrosis and Tay-Sachs disease who were anonymously tested for *BRCA1* mutations, carried the 185delAG *BRCA1* mutation.[6]

To assess the frequency of the 185delAG mutation in Jewish women with early-onset breast cancer, we tested DNA samples from 107 Ashkenazi women with breast cancer seen at our institution.

Methods

We studied two groups of probands. The first group consisted of women who were diagnosed as having breast cancer before age 42 years; family history of breast cancer could be positive or negative in this group. In the second group, the probands had breast cancer diagnosed at or before age 50 and satisfied one of the following criteria: one or more first-degree relatives had breast or ovarian cancer diagnosed at or before age 50; two or more second-degree relatives had breast or ovarian cancer with at least one relative being diagnosed at or before age 50; or the proband had ovarian as well as breast cancer diagnosed before age 50.

Participants for the study were found through medical oncology and genetic counselling clinics, and all eligible patients were asked to take part in the study. A family history was obtained by a self-report questionnaire. Histological confirmation of diagnosis was obtained for all probands. Religious background was confirmed for all probands by self report or interview. All participants gave informed consent for genetic studies. For participants who indicated interest in receiving test results as part of a second stage of consent the test results will be confirmed independently by sequencing; and they will be informed of their results after genetic counselling. All participants were given the option not to know their test results. Extensive genetic counselling, covering options for detection and prevention, is available irrespective of the decision on whether or not to learn the results of DNA testing.

185delAg mutations of *BRCA1* were detected by amplification of genomic DNA according to standard polymerase chain reaction (PCR) procedures.[7,8] The following primers were added to the 10 μL of reaction mixture which contained 20 ng DNA: 185D-R(CGTTGAAGAAGTACAAAATGTCA) and 185D-L(TCAATTCTGTTCATTTGCATAGG). The reaction

Group	Number tested	185delAG positive	
		Number positive	% (95% CI)
Diagnosis before age 42 years			
Overall	80	16	20 (11–29)
Diagnosed before age 37 years	40	7	18 (6–29)
Diagosed age 37–41 years	40	9	22 (10–35)
First-degree relative affected	31	11	35 (19–52)
No first-degree relative affected	49	5	10 (2–19)
Diagnosis ages 42–50 years and positive family history*	27	8	30 (12–47)

*Family history for this group was defined as one first-degree or two second-degree relatives diagnosed with breast or ovarian cancer, one before age 50 years. One proband with both breast and ovarian cancer was included in this cohort.

Table: **Occurrence of 185delAG mutation in breast cancer patients**

mixture and primers were annealed at 55°C. The PCR product sizes were 130 bp for the non-deleted segment and 128 bp for the section with the 185delAG 2 bp deletion. The radiolabelled PCR products were separated by electrophoresis on standard 6% polyacrylamide denaturing sequencing gels[10] at 65 W for 2 h. The gels were then dried and autoradiographed. In several cases with a 128 bp fragment, the fragment was sequenced to confirm the presence of the 185delAG mutation. A positive control was included on each gel.

Results

The frequencies of the 185delAG mutation in the two groups are shown in the table. 16 (20%) of 80 patients who presented with early-onset (before age 42 years) breast cancer were heterozygous for the 185delAG mutation. As predicted, the prevalence of this type of mutation was significantly higher in the subset of patients with a first-degree relative who had breast cancer or ovarian cancer ($p=0·006$). The five 185delAG heterozygotes in the group whose first-degree relatives were free of breast cancer each had one or more second-degree relatives with breast cancer. Half of the probands were diagnosed as having breast cancer before the age of 37 years. Although we found no significant difference in the frequency of the 185delAG mutation between those who presented before and after the age of 37 years, the power to detect a rate or frequency difference that was anything less than four-fold was quite low.

8 (30%) of 27 probands who presented with breast cancer between the ages of 42 and 50 years who had a positive family history, were heterozygous for the 185delAG mutation. If we combine all the patients from the two groups who had at least one first-degree relative with breast or ovarian cancer of the 57 patients 19 (33%, 95% CI 20–44) were heterozygous for 185delAG.

In 18 families there were one or more cases of ovarian cancer in first-degree or second-degree relatives of the proband, or in two cases, the proband herself. Among this subset, ten (56%) families had the 185delAG deletion. Overall, ten (42%) of the 24 probands with 185delAG mutations had a family, or personal, history of ovarian cancer compared with 8 (10%) of 83 of probands without this mutation ($p<0·001$).

Discussion

Struewing and colleagues[9] studied 858 Ashkenazi Jews and estimated a 0·9% carrier frequency of the 185delAG mutation. On the basis of estimates of *BRCA1*-associated risk from family studies, they predicted that 16% of breast-cancer cases diagnosed before age 50 years among Ashkenazi Jewish women would be due to the 185delAG mutation.

With the same approach and with data from Struewing and colleagues' study, we estimated that 22% of the 80 women in our study group with early-onset (before age 42) breast cancer would have been attributable to 185delAG. The calculated proportion is only slightly higher than the 20% observed in our study, and well within its 95% confidence limits. However, the rate observed in a population-based series may be lower because of the bias toward probands with family histories of cancer seen in our clinics. If the true proportion due to 185delAG is lower than that predicted by Struewing and colleagues either the carrier frequency must be lower than 0·9% or the penetrance of 185delAG is lower than pooled estimates of multiple *BRCA1* mutations obtained from linkage studies of high-risk families. In addition, the mutation frequency seemed to be similar in the groups diagnosed at ages 37 to 41 years and diagnosed before the age of 37 years. This finding is not consistent with predictions of a higher relative risk associated with younger age at onset,[1,2] although these studies have typically focused on much broader age classifications. Since the sample size in our study is small, the relative risk in women diagnosed with breast cancer at younger age may, in fact, be higher, and our results may reflect chance variation.

Follow-up of women who are heterozygous for the mutation will allow more precise estimates of age-specific cancer risks. Further studies of this mutation in postmenopausal women, ascertained without regard to family history, will be necessary to define its frequency.

All of the 185delAG heterozygotes identified in this study had a family history of the disease. This finding has implications for preventive oncology management, since, with the exception of age, family history remains the single most powerful predictor of breast-cancer risk for women. All of the five 185delAG heterozygotes with no first-degree relative with breast or ovarian cancer had an affected second-degree relative (four paternal, one maternal).

The clinical value of a negative test for the 185delAG mutation even in the Ashkenazi population is limited, because only 32% of families who had at least two members with early-onset breast cancer had the mutation. Similarly more than 40% of the women with a first-degree or second-degree relative with ovarian cancer did not have the 185delAG mutation. These observations should be taken into account in counselling. The remaining familial cases could be due to other mutations in *BRCA1*, mutations in *BRCA2* and other high-penetrance genes, or variation in more common low-penetrance genes.

Because of the frequency of the 185delAG both in the general population of Ashkenazi Jews and in Jewish women with early-onset breast cancer or with a family history of the disease, screening for this mutation of *BRCA1* may serve as a useful tool in the genetic counselling of these families.

This study was supported by grants P20 CA 58232, RO1CA65673, and RO1 CA55914 from the National Cancer Institute, grant DAMD 17-94-JH260 from the Department of Defense, and the Dewitt Wallace Fund. We thank Lucio Luzzatto for his critical reading of the manuscript.

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The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds

S.V. Tavtigian[1], J. Simard[2], J. Rommens[3], F. Couch[4], D. Shattuck-Eidens[1], S. Neuhausen[5], S. Merajver[6], S. Thorlacius[7], K. Offit[8], D. Stoppa-Lyonnet[9], C. Belanger[2], R. Bell[1], S. Berry[1], R. Bogden[1], Q. Chen[1], T. Davis[1], M. Dumont[2], C. Frye[1], T. Hattier[1], S. Jammulapati[1], T. Janecki[1], P. Jiang[1], R. Kehrer[1], J.-F. Leblanc[2], J.T. Mitchell[1], J. McArthur-Morrison[3], K. Nguyen[5], Y. Peng[4], C. Samson[2], M. Schroeder[1], S.C. Snyder[1], L. Steele[5], M. Stringfellow[1], C. Stroup[1], B. Swedlund[1], J. Swensen[5], D. Teng[1], A. Thomas[1], T. Tran[1], T. Tran[5], M. Tranchant[2], J. Weaver-Feldhaus[1], A.K.C. Wong[1], H. Shizuya[10], J.E. Eyfjord[7], L. Cannon-Albright[5], F. Labrie[2], M.H. Skolnick[1,5], B. Weber[4], A. Kamb[1] & D.E. Goldgar[5,11]

[1]*Myriad Genetics Inc., Salt Lake City, Utah USA*
[2]*Laboratory of Molecular Endocrinology, CHUL Research Center and Laval University, Quebec City, Canada*
[3]*Department of Genetics, Hospital for Sick Children, University of Toronto, Ontario, Canada*
[4]*Deparment of Hematology-Oncology, University of Pennsylvania, Philadelphia, USA*
[5]*Genetic Epidemiology Group, Department of Medical Informatics and Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, Utah, USA*
[6]*Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA*
[7]*Molecular and Cell Biology Research Laboratory, Icelandic Cancer Society, Rekjavik, Iceland*
[8]*Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York, New York, USA*
[9]*Unite de Genetique Oncologique, Institute Curie, Paris, France*
[10]*Division of Biology, California Institute of Technology, Pasadena, California, USA*
[11]*Unit of Genetic Epidemiology, International Agency for Research on Cancer, 150 cours Albert Thomas, 63972, Lyon, Cedex 08, France*

Correspondence should be addressed to D.E.G.[11]

Breast carcinoma is the most common malignancy among women in developed countries. Because family history remains the strongest single predictor of breast cancer risk, attention has focused on the role of highly penetrant, dominantly inherited genes in cancer-prone kindreds[1]. *BRCA1* was localized to chromosome 17 through analysis of a set of high-risk kindreds[2], and then identified four years later by a positional cloning strategy[3]. *BRCA2* was mapped to chromosomal 13q at about the same time[4]. Just fifteen months later, Wooster *et al.*[5] reported a partial *BRCA2* sequence and six mutations predicted to cause truncation of the BRCA2 protein. While these findings provide strong evidence that the identified gene corresponds to *BRCA2*, only two thirds of the coding sequence and 8 out of 27 exons were isolated and screened; consequently, several questions remained unanswered regarding the nature of *BRCA2* and the frequency of mutations in 13q-linked families. We have now determined the complete coding sequence and exonic structure of *BRCA2* (GenBank accession #U43746), and examined its pattern of expression. Here, we provide sequences for a set of PCR primers sufficient to screen the entire coding sequence of *BRCA2* using genomic DNA. We also report a mutational analysis of *BRCA2* in families selected on the basis of linkage analysis and/or the presence of one or more cases of male breast cancer. Togetner with the specific mutations described previously, our data provide preliminary insight into the *BRCA2* mutation profile.

BRCA2 lies near the centre of a 1.4-megabase (Mb) interval flanked by markers *D13S1444* and *D13S310* (F.C. *et al.*, unpublished), completely within a 0.3-Mb homozygous deletion identified in a pancreatic carcinoma xenograft[6]. The full-length sequence of the *BRCA2* transcript was assembled by combination of several smaller sequences obtained from hybrid selection, exon trapping, cDNA library screening, genomic sequencing and inter-clone PCR experiments using cDNA as template for amplification ('island hopping'; Fig. 1*a*). The extreme 5' end of the mRNA, including the predicted translational start site, was identified by a modified 5' RACE protocol[7]. The first nucleotide in the sequence (nt 1) is a non-template G, an indication that the mRNA cap is contained in the sequence. A portion of exon 11, which is nearly 5 kilobases (kb) in length, was identified by analysis of roughly 900 kb of genomic sequence in the public domain (ftp://genome.wust1.edu/pub/gscl/brca). This genomic sequence was condensed with our own genomic sequence into a set of 160 sequence contigs. When the condensed sequence was scanned for open reading frames (ORFs), a contiguous stretch of nearly 5 kb was identified which was spanned by long ORFs. This sequence was linked together by island hopping experiments with two previously identified candidate gene fragments (F.C. *et al.*, unpublished)[8]. Our composite *BRCA2* cDNA sequence consists of 11,385 bp but does not include the polyadenylation signal or poly(A) tail.

Conceptual translation of the cDNA reveals an ORF beginning at nt 229 and encoding a protein of 3,418 amino acids. The peptide bears no similarity to other proteins apart from sequence composition. There is no signal sequence at the N terminus, and no obvious membrane-spanning regions. Like BRCA1, the BRCA2 protein is highly charged; roughly one quarter of the residues are acidic or basic. However, there are few clues as to the biochemical function of BRCA2.

The *BRCA2* gene structure was determined by comparing cDNA and genomic sequences. *BRCA2* is composed of 27 exons distributed over roughly 70 kb of genomic DNA (Fig. 1*b*). A CpG-rich region at the 5' end of *BRCA2* extending upstream suggests the presence of regulatory signals often associated with CpG 'islands.' Unlike most human genes, the coding sequence is AT-rich (> 60%). Based on Southern blot experiments, *BRCA2* appears to be unique, with no close homologue in the human genome (data not shown).

Hybridization of labelled cDNA to human multiple tissue northern filters revealed an 11–12 kb transcript detectable in thymus and testis (Fig. 2*a*), suggesting that little of the *BRCA2* mRNA sequence is missing from our composite cDNA. Because the northerns did not include mammary gland RNA, we performed RT-PCR experiments using a *BRCA2* cDNA amplicon that spans the last splice junction on a set of human tissue RNAs (Fig. 2b). All of the samples produced positive signals. The highest levels of expression were observed in breast and thymus, with slightly lower levels in lung, ovary and spleen. This pattern of expression is similar to that produced by *BRCA1* amplicons[3].

Individuals from 18 putative *BRCA2* kindreds were screened for *BRCA2* germline mutations by DNA sequence analysis[4]. Twelve kindreds have at least one case of male breast cancer; four have two or more cases; and four include at least one individual affected with ovarian cancer who shares the linked *BRCA2* haplotype. Each of the 18 kindreds has a posterior probability of harboring a *BRCA2* mutation of at least 69% and 9 kindreds have probabilities greater than 90%. Based on these combined probabilities, 16 of the 18 kindreds are expected to segregate *BRCA2* mutations. The entire coding sequence and associated splice junctions were

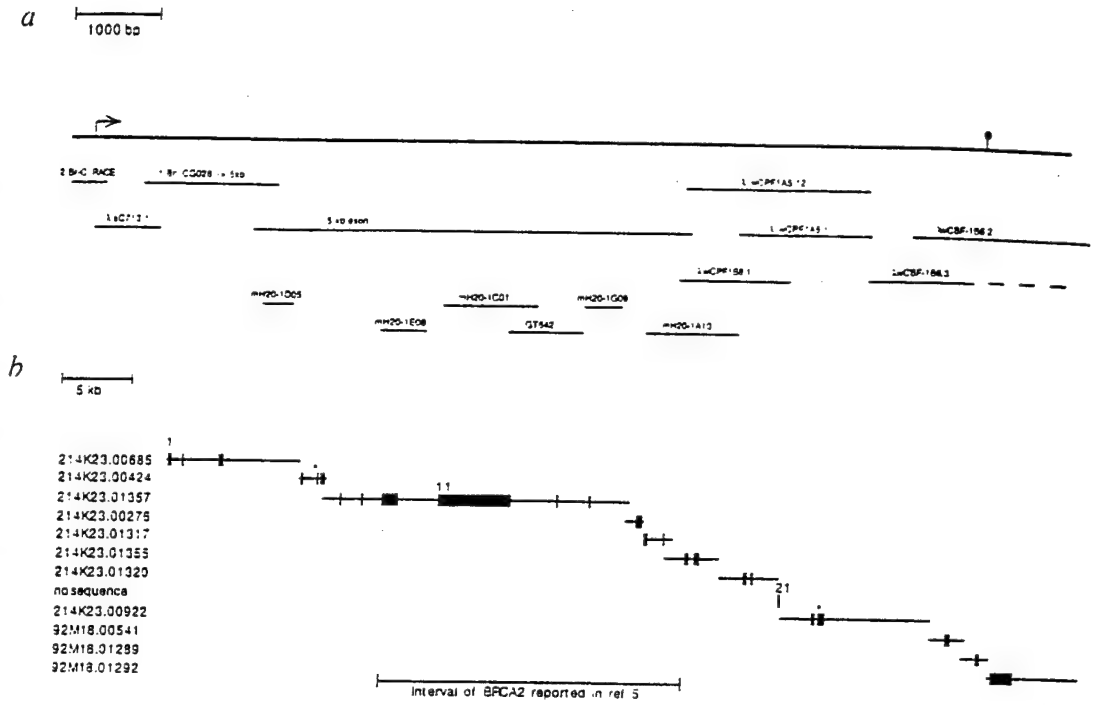

Fig. 1 a, Cloning of *BRCA2*. Sequence-space relationships between the cDNA clones, hybrid selected clones, cDNA PCR products, and genomic sequences used to assemble the *BRCA2* transcript sequence. 2-Br-C:RACE is a biotin-capture RACE product obtained from both human breast and human thymus cDNA. The cDNA clone λ sC713.1 was identified by screening a pool of human testis and HepG2 cDNA libraries with hybrid selected clone GT 713. The sequence 1-Br:CG026—5 kb was generated from a PCR product beginning at the exon 7/8 junction (within λ sC713.1) and terminating within an hybrid selected clone that is part of exon 11. The sequence of exon 11 was corrected by comparison to hybrid selected clones, genomic sequence in the public domain, and radioactive DNA sequencing gels. Hybrid selected clones located within that exon (clone names beginning with mH or GT) are placed below it. The cDNA clones λ wCPF1B8.1, λ wCPF1A5.1, λ wCPF1A5.12, λ wCBF1B6.2, and λ wCBF1B6.3 were identified by screening a pool of human mammary gland, placenta, testis, and HepG2 cDNA libraries with the exon trapped clones wXBF1B8, wXPF1A5, and wXBF1B6. The clone λ wCBF1B6.3 is chimaeric (indicated by the dashed line), but its 5' end contained an important overlap with λ wCPF1A5.1. ↱ denotes the translation initiator. * denotes the translation terminator. *b*, Genomic organization of *BRCA2*. The exons (boxes and/or vertical lines) are parsed across the publicly available genomic sequences (horizontal lines) such that their sizes and spacing are proportional. The name of each genomic sequence is given on the left. The sequences 92M18.00541 and 92M18.01289 actually overlap. Distances between the other genomic sequences are not known. No databases contained genomic sequences overlapping with exon 21. The extent of the peptide sequence published by Wooster *et al.*[5] is indicated below the parsed exons. Exons 1, 11 and 21 are numbered. * denotes two adjacent exons spaced closely enough that they are not resolved at this scale.

screened for mutations in multiple individuals from 9 kindreds using either cDNA or genomic DNA (Table 1*a*). Individuals from the remaining 9 kindreds were screened for mutations using only genomic DNA (Table 1*b*). These latter screening experiments encompassed 99% of the coding sequence (all exons excluding exon 15) and all but two of the splice junctions.

We identified potentially deleterious sequence alterations in 9 of the 18 kindreds (Table 1). All except one — a deletion of three nucleotides (kindred 1019) — involved nucleotide deletions that altered the reading frame, leading to truncation of the BRCA2 protein. The 3-nt deletion was not observed in 36 unrelated breast cancer cases, hence we have included it in our mutation tally although its effect on BRCA2 function must be proved. All 9 mutations are distinct. In most cases, segregation studies show that the mutations are present in multiple haplotype carriers and absent in noncarriers (data not shown). In addition to these mutations, three silent and three missense substitutions were detected. Based on their frequencies in a set of control chromosomes, we have classified these variants as neutral polymorphisms (Table 1*c*).

Nine of the 18 kindreds were tested for transcript loss. Specific polymorphic sites known to be heterozygous in genomic DNA (Table 1*c*) were examined in cDNA from kindred individuals. The appearance of hemizygosity was interpreted as evidence for a mutation leading to reduction in mRNA levels. Two of the 9 kindreds displayed signs of reduced transcript levels. However, one of these kindreds (1018) contained a previously identified frameshift mutation, while the second (2367) contained an aberrantly spliced *BRCA2* mRNA that lacked exon 2 (data not shown). The abundance of this mutant transcript was estimated at roughly 20% of wild-type. This implies that some mutations in the *BRCA2* coding sequence may destabilize the transcript in addition to disrupting the protein sequence, similar to *BRCA1* (ref. 9). In no case was a purely regulatory mutation inferred. In summary, 56% of the kindreds (10/18) contained an altered *BRCA2* gene. Half of our kindreds contained microdeletion mutations, mostly frameshifts; none contained missense or nonsense mutations.

BRCA2 is remarkably similar to *BRCA1*. Both genes encode exceptionally large, highly charged proteins; both have many exons; both have a large exon 11 (3,426 bp for *BRCA1* and 4,932 bp for *BRCA2*); both have translational start sites in exon 2; both have coding sequences that are AT-rich; both span approximately 70 kb of genomic DNA; and, both are expressed at high levels in testis. Whether or not *BRCA1* and *BRCA2* participate in the same pathway of tumour suppression in breast epithelium is not known. The different phenotypes of the two mutant genes, particularly the role of *BRCA2* in male breast cancer, suggest that they may not function in the same genetic pathway.

Mutational analysis of *BRCA2* reveals other features in common with *BRCA1*. The distribution of mutations in *BRCA2* appears to be uniform based on our data and data from Wooster *et al.*[5]. Mutations have been identified in 6 of the 26 coding exons of *BRCA2* (exons 2, 9, 10, 11, 18 and 23). Nine mutations have been detected

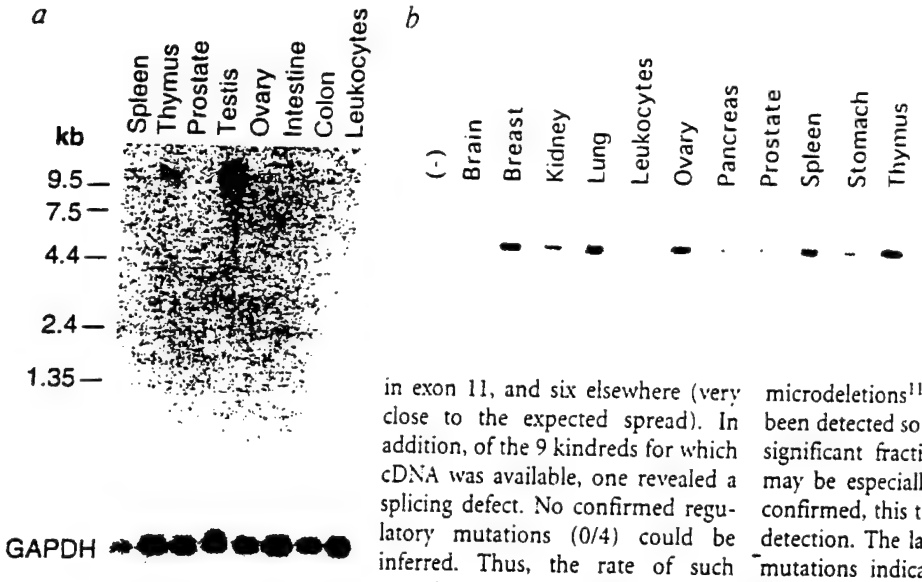

Fig. 2 *a*, Analysis of *BRCA2* expression. Upper panel: Multiple tissue northern (MTN) filters (Clontech) probed with the 1.55-kb 1-Br:CG026→5-kb PCR product. The 11–12 kb *BRCA2* transcript is detected in testis. Lower panel: the same filter probed with glyceraldehyde-3-phosphate dehydrogenase. *b*, RT-PCR analysis of *BRCA2* expression. A BRCA2 cDNA amplicon spanning the last splice junction was amplified from 0.4 ng or random-primed A^+ cDNA from the indicated tissues.

in exon 11, and six elsewhere (very close to the expected spread). In addition, of the 9 kindreds for which cDNA was available, one revealed a splicing defect. No confirmed regulatory mutations (0/4) could be inferred. Thus, the rate of such mutations in *BRCA2* appears to be comparable (or lower) to the rate observed in *BRCA1* (10–20%)[10].

The mutation profile of *BRCA2*, however, may differ from *BRCA1*. Of the 15 sequence alterations described so far in *BRCA2* (9 here, 6 from ref. 5), all involve deletions of 1–6 nucleotides. In contrast, microinsertions plus point mutations in *BRCA1* are about as common as microdeletions[11]. Furthermore, no point mutations have been detected so far in *BRCA2*, whereas they constitute a significant fraction of *BRCA1* variants. Thus, *BRCA2* may be especially vulnerable to deletion mutations. If confirmed, this trait has clear implications for mutation detection. The lack of multiple observations of specific mutations indicates that the number of independent *BRCA2* mutations in the population may be even greater than the number of *BRCA1* mutations.

Approximately two thirds as many mutations were detected in *BRCA2* as expected among our 18 families. This may imply the presence of mutations in regions that are difficult to detect, or in regions that were not screened. For example, only half of the kindreds were screened for regulatory mutations due to lack of cDNA

Table 1 *BRCA2* mutations and polymorphisms

a, Families screened for complete coding sequence (informative cDNA sample)

Family	Number of cancer cases				Lod	Prior	*BRCA2*			
	FBC	FBC <50yrs	Ov	MBC	score	probability	mutation	exon	codon	effect
UT-107	20	18	2	3	5.06	1.00	277 delAC	2	17	termination codon at 29
UT-1018	11	9	0	1	2.47	1.00	982 del4	9	252	termination codon at 275
UT-2044	8	6	4	1	2.13	1.00	4706 del4	11	1493	termination codon at 1502
UT-2367	9	5	1	0	2.09	0.99	SP			deletion of exon 2 in mRNA
UT-2327	13	6	0	0	1.92	0.99	ND			
UT-2388	3	3	1	0	0.92	0.92	ND			
UT-2328	10	4	0	1	0.21	0.87	ND			
UT-4328	4	3	0	0	0.18	0.69	ND			
MI-1016	4	2	0	1	0.04	0.81	ND			

b, Families screened for all exons except 15 (no cDNA sample available)

CU-20	4	3	2	2	1.09	1	8525 delC	18	2766	termination codon at 2776
CU-159	8	4	0	0	0.99	0.94	9254 del5	23	3009	termination codon at 3015
UT-2043	2	2	1	1	0.86	0.97	4075 delGT	11	1283	termination codon at 1285
IC-2204	3	1	0	4	0.51	0.98	999 del5	9	257	termination codon at 273
MS-075	4	1	0	1	0.50	0.93	6174 delT	11	1982	termination codon at 2003
UT-1019	5	1	0	2	nd	0.95	4132 del3	11	1302	deletion of thr1302
UT-2027	4	4	0	0	0.39	0.79	ND			
MS-036	3	2	0	1	nd	0.90	ND			
UT-2171	5	4	2	0	nd	nd	ND			

c, Common polymorphisms in *BRCA2*

Polymorphism	Description	Effect	Number of chromosomes A	C	G	T	Total
5'UTR-203	TACCAA(G/A)CATTG		9	0	25	0	34
PM-1342	GTA GCA (C/A)AT CAG	His→Asn	24	14	0	0	38
PM-2457	GTA CAA CA(T/C) TCA	His→His	2	0	0	38	40
PM-3199	TAC ATG (A/G)AC AAT	Asn→Asp	37	0	3	0	40
PM-3668	CCT GAA A(A/G)C CAG	Asn→Ser	34	0	6	0	40
PM-4035	GAT TCT GT(T/C) GTT	Val→Val	0	4	0	36	40
PM-7470	ACT AAA TC(A/G) CAT	Ser→Ser	34	0	6	0	40
3'UTR-10,854	AAAAGAA(G/A)CATTTCTA		17	0	15	0	32
3'UTR-11,316	ATTTATTTTTTTT(T)CAAC	T9 vs. T10	T9: (7)		T10: (29)		36

Mutations and polymorphisms are given by nucleotide position. SP–inferred splice mutation. ND–none detected. nd–not determined. FBC–female breast cancer. Ov–ovaian cancer. MBC–male breast cancer.

Table 2 Primers used to amplify and mutation screen *BRCA2* from genomic DNA

Exon	Forward Primer	Reverse Primer	Nested Primer
Exon 2	TGTTCCCATCCTCACAGTAAG*	GTACTGGGTTTTTAGCAAGCA*	
Exon 3	GGTTAAAACTAAGGTGGGA*	ATTTGCCCAGCATGACACA*	
Exon 4	TTTCCCAGTATAGAGGAGA*	GTAGGAAAATGTTTCATTTAA*	
Exon 5	ATCTAAAGTAGTATTCCAACA*	GGGGGTAAAAAAAGGGGAA*	
Exon 6	GAGATAAGTCAGGTATGATT*	AATTGCCTGTATGAGGCAGA*	
Exon 7	GGCAATTCAGTAAACGTTAA*	ATTGTCAGTTACTAACACAC*	
Exon 8	GTGTCATGTAATCAAATAGT*	CAGGTTTAGAGACTTTCTC*	
Exon 9	GGACCTAGGTTGATTGCA*	GTCAAGAAAGGTAAGGTAA*	
Exon 10-1	CTATGAGAAAGGTTGTGAG*	CCTAGTCTTGCTAGTTCTT*	
Exon 10-2	AACAGTTGTAGATACCTCTGAA*	GACTTTTTGATACCCTGAAATG*	
Exon 10-3	CAGCATCTTGAATCTCATACAG*	CATGTATACAGATGATGCCTAAG*	
Exon 11-1	AACTTAGTGAAAAATATTTAGTGA	ATACATCTTGATTCTTTTCCAT*	TTTAGTGAATGTGATTGATGGT*
Exon 11-2	AGAACCAACTTTGTCCTTAA	TTAGATTTGTGTTTTGGTTGAA*	TAGCTCTTTTGGGACAATTC*
Exon 11-3	ATGGAAAAGAATCAAGATGTAT*	CCTAATGTTATGTTCAGAGAG	GCTACCTCCAAAACTGTGA*
Exon 11-4	GTGTAAAGCAGCATATAAAAAT*	GTTGCTGCTGTCTACCTG	AGTGGTCTTAAGATAGTCAT*
Exon 11-5	CCATAATTTAACACCTAGCCA**	CCAAAAAAGTTAAATCTGACA**	
	GGCTTTTATTCTGCTCATGGC*	CCTCTGCAGAAGTTTCCTCAC*	
Exon 11-6	AACGGACTTGCTATTTACTGA*	AGTACCTTGCTCTTTTTCATC*	
Exon 11-7	CAGCTAGCGGGAAAAAAGTTA*	TTCGGAGAGATGATTTTTGTC*	
Exon 11-8	GCCTTAGCTTTTTACACAA*	TTTTTGATTATATCTCGTTG	TTATTCTCGTTGTTTTCCTTA*
Exon 11-9	CCATTAAATTGTCCATATCTA*	GACGTAGGTGAATAGTGAAGA	TCAAATTCCTCTAACACTCC*
Exon 11-10	GAAGATAGTACCAAGCAAGTC	TGAGACTTTGGTTCCTAATAC*	AGTAACGAACATTCAGACCAG*
Exon 11-11	GTCTTCACTATTCACCTACG*	CCCCCAAACTGACTACACAA	AGCATACCAAGTCTACTGAAT*
Exon 12	ACTCTTTCAAACATTAGGTCA*	TTGGAGAGGCAGGTGGAT	CTATAGAGGGAGAACAGAT*
Exon 13	TTTATGCTGATTTCTGTTGTAT	ATAAAACGGGAAGTGTTAACT*	CTGTGAGTTATTTGGTGCAT*
Exon 14	GAATACAAAACAGTTACCAGA	CACCACCAAAGGGGGAAA*	AAATGAGGGTCTGCAACAAA*
Exon 15	ATTTCAATTTTATTTTTGCT*	ATGAAATAAAATTACACTCTGTC	TACACTCTGTCATAAAAGCC*
Exon 16	ATGTTTTTGTAGTGAAGATTCT	TAGTTCGAGAGACAGTTAAG*	CAGTTTTGGTTTGTTATAATTG*
Exon 17	CAGAGAATAGTTGTAGTTGTT	AACCTTAACCCATACTGCC*	TTCAGTATCATCCTATGTGG*
Exon 18	TTTTATTCTCAGTTATTCAGTG	GAAATTGAGCATCCTTAGTAA*	AATTCTAGAGTCACACTTCC*
Exon 19	ATATTTTTAAGGCAGTTCTAGA	TTACACACCAAAAAAGTCA*	TGAAAACTCTTATGATATCTGT*
Exon 20	TGAATGTTATATATGTGACTTTT*	GTTGTTGCTATTCTTTGTCTA	CCCTAGATACTAAAAAATAAAG*
Exon 21	CTTTTAGCAGTTATATAGTTTC	GCCAGAGAGTCTAAAACAG*	CTTTGGGTGTTTTATGCTTG*
Exon 22	TTTGTTGTATTTGTCCTGTTTA	ATTTTGTTAGTAAGGTCATTTTT*	GTTCTGATTGCTTTTTATTCC*
Exon 23	ATCACTTCTTCCATTGCATC*	CCGTGGGCTGGTAAATCTG*	
Exon 24	CTGGTAGCTCCAACTAATC*	ACCGGTACAAACCTTTCATTG*	
Exon 25	CTATTTTGATTTGCTTTTATTATT*	GCTATTTCCTTGATACTGGAC*	
Exon 26	TTGGAAACATAAATATGTGGG*	ACTTACAGGAGCCACATAAC*	
Exon 27	CTACATAATTATGATAGGCTNCG**	GTACTAATGTGTGGTTTGAAA**	
		TCAATGCAAGTTCTTCGTCAGC*	

Primers with a * were used for sequencing.
Primers without a * are replaced by the internal nested primer in the third column for both the second round of PCR and sequencing. For large exons requiring internal sequencing primers, ** signifies the primers used to amplify the exon.

samples. In addition, in only one of the two families in which transcript loss was detected was the actual sequence alteration identified. However, 7/9 kindreds with Lod scores over 0.39 revealed mutations. Thus, it is possible that the assumed prior probabilities for our kindreds were inflated. Several of the kindreds for which mutations have not been defined may not segregate *BRCA2* mutations. Many have been screened without success for *BRCA1* mutations. Therefore, some of the families may represent sporadic clusters; others may be afflicted with breast cancer due to segregation of genes besides *BRCA1* and *BRCA2*.

The characterization of two genes, *BRCA1* and *BRCA2*, that together may account for the vast majority of early-onset hereditary breast cancer, is a major step toward early detection of an important human disease. One of the significant goals ahead is the development of reliable diagnostic tests for *BRCA1* and *BRCA2*. In addition, the definition of other genes that may contribute to breast cancer incidence is an important pursuit.

Methods

Hybrid selection. Two distinct methods of hybrid selection were used. ***Method 1: cDNA preparation and selection.*** Poly(A)$^+$ enriched RNA from human mammary gland, ovary, testis, fetal brain and placenta tissues and from total RNA of the cell line Caco-2 (ATCC HTB 37) were reverse transcribed using the tailed random primer $RXGN_9$ (5'–CGGAATTCTGCAGATCTA'B'C'N_9) and M-MLV Reverse Transcriptase (Life Technologies, Inc.). First strand cDNA was poly(A) tailed, 2nd strand synthesis was primed with the oligo $RXGT_{12}$ (5'–CGGAATTCTGCAGATCT$_{12}$), and then the ds cDNA was expanded by amplification with the primer RXG (5'–CGGAATTCTGCAGATCT). Hybrid selection was carried out for two consecutive rounds to immobilized P1 or BAC DNA as described[12,13]. Groups of two to four overlapping P1 and/or BAC clones were used in individual selection experiments. Hybridizing cDNA was collected, passed over a G30 Fine Sephadex column and reamplified using the primer RXG. The products were then digested with *Eco*RI, size selected on agarose gels, and ligated into pBluescript (Stratagene) that had been digested with *Eco*RI and treated with calf alkaline phosphatase (Boehringer Mannheim). Ligation products were transformed into competent DH5α *E.coli* cells (Life Technologies, Inc.). *Characterization of retrieved cDNAs.* 200 to 300 individual colonies from each ligation (from each 250 kb of genomic DNA) were picked and gridded into microtitre plates for ordering and storage. Cultures were replica transferred onto Hybond N membranes (Amersham) supported by LB agar with ampicilin. Colonies were allowed to propagate and were subsequently lysed with standard procedures. Initial analysis of the cDNA clones involved a prescreen for ribosomal sequences and subsequent cross screenings for detection of overlap and redundancy. Approximately 10–25% of the clones were eliminated as they hybridized strongly with radiolabelled cDNA obtained from total RNA. Plasmids from 25 to 50 clones from each selection experiment that did not hybridize in the prescreening were isolated for further analysis. The retrieved cDNA fragments were verified to originate from individual starting genomic clones by hybridization to restriction digests of DNAs of the starting clones, of a hamster hybrid cell line (GM10898A) that contains chromosome 13 as its only human material and to human genomic DNA. The clones were tentatively assigned into groups based on the overlapping or nonoverlapping intervals of the genomic clones. Of clones tested, approximately 85% mapped appropriately to the starting clones.

Method 2: (refs 14, 15): *cDNA preparation.* Poly(A)$^+$ enriched RNA from human mammary gland, brain, lymphocyte, and stomach were reverse transcribed using the tailed random primer XN_{12} [5'–(NH_2)-GTAGTGCAAGGCTCGAGAACN$_{12}$] and Superscript II reverse transcriptase (Gibco BRL). After 2nd strand synthesis and end polishing, the ds cDNA was purified on sepahrose CL-4B columns (Pharmacia). cDNAs were 'anchored' by ligation of a double stranded oligo RP [5'–(NH2)-TGAGTAGAATTCTAACGGCCGTCATTGTTC annealed to 5'–GAACAATGACGGCCGTTAGAATTCTAC-TCA-(NH_2)] to their 5' ends (5' relative to mRNA) using T4 DNA ligase. Anchored ds cDNA was then repurified on sepahrose CL-4B columns. *Selection.* cDNAs from mammary gland, brain, lymphocyte, and stomach tissues were first amplified using a nested version of RP (RP.A: 5'–TGAGTAGAATTC-TAACGGCCGTCAT) and XPCR [5'–(PO4)-GTAGTGCAAG-GCTCGAGAAC] and purified by fractionation on Sepharose CL-4B. Selection probes were prepared from purified P1s, BACs, or PACs, by digestion with *Hint*I and Exonuclease III. The single stranded probe was photo-labelled with photobiotin (Gibco BRL) according to the manufacturers recommendations. Probe, cDNA and Cot-1 DNA were hybridized in 2.4 M TEA-Cl, 10mM NaPO4, 1mM EDTA. Hybridized cDNAs were captured on streptavidin-paramagnetic particles (Dynal), eluted, reamplified with a further nested version of RP [RP.B: 5'–(PO4)-TGAGTA-GAATTCTAACGGCCGTCATTG] and XPCR, and size selected on Sepharose CL-6B. The selected, amplified cDNA was hybridized with an additional aliquot of probe and Cot-1 DNA. Captured and eluted products were amplified again with RP.B and XPCR, size selected by gel electrophoresis and cloned into dephosphorylated *Hinc*II-cut pUC18. Ligation products were transformed into XL2-Blue ultra-competent cells (Stratagene). *Analysis.* Approximately 192 colonies for each single-probe selection experiment were amplified by colony PCR using vector primers and blotted in duplicate onto Zeta Probe nylon filters (Bio-Rad). The filters were hybridized using standard procedures with either random primed Cot-1 DNA or probe DNA (P1, BAC, or PAC). Probe positive, Cot-1 negative clones were sequenced in both directions using vector primers on an ABI 377 sequencer.

Exon trapping. Exon amplification was performed using a minimally overlapping set of BACs, P1s and PACs in order to isolate a number of gene sequences from the *BRCA2* candidate region.

Pools of genomic clones were assembled, containing from 100–300 kb of DNA in the form of 1-3 overlapping genomic clones. Genomic clones were digested with *Pst*I or *Bam*H1 + *Bgl*11 and ligated into *Pst*I or *Bam*H1 sites of the pSPL3 splicing vector. The exon amplification technique was performed[16] and the end products were cloned in the pAMP1 plasmid from the Uracil DNA Glycosylase cloning system (BRL). Approximately 6,000 clones were picked, propagated in 96 well plates, stamped onto filters, and analysed for the presence of vector and repeat sequences by hybridization. Each clone insert was PCR amplified and tested for redundancy, localization, and human specificity by hybridization to grids of exons and dot blots of the parent genomic DNA. Unique candidate exons were sequenced, searched against the databases, and used for hybridization to cDNA libraries.

5' RACE. The 5' end of *BRCA2* was identified by a modified RACE protocol called biotin capture RACE. Poly(A)$^+$ enriched RNA from human mammary gland and thymus was reverse transcribed using the tailed random primer XN_{12} [5'–(NH_2)-GTAGTGCAAG-GCTCGAGAACN_{12}-3'] and Superscript II reverse transcriptase (Gibco BRL). The RNA strand was hydrolysed in NaOH and first strand cDNA purified by fractionation on Sepharose CL-4B (Pharmacia). First strand cDNAs were 'anchored' by ligation of a double stranded oligo with a 7 bp random 5' overhang [ds UCA: 5'–CCTTCACACGCGTATCGATTAGTCACN$_7$–(NH_2) annealed to 5'(PO_4)-GTGACTAATCGATACGCGTGTGAAGGTGC] to their 3' ends using T4 DNA ligase. After ligation, the anchored cDNA was repurified by fractionation on Sepharose CL-4B. The 5' end of BRCA2 was amplified using a biotinylated reverse primer [5'(B)-TTGAAGAACAACAGGACTTTCACTA] and a nested version of UCA [UCP.A: 5'–CACCTTCACACGCGTATCG]. PCR products were fractionated on an agarose gel, gel purified, and captured on streptavidin-paramagnetic particles (Dynal). Captured cDNA was reamplified using a nested reverse primer [5'–GTTCGTAATTGTTGTTTTTATGTTCAG] and a further nested version of UCA [UCP.B: 5'–CCTTCACACGCGTATC-GATTAG]. This PCR reaction gave a single sharp band on an agarose gel: the DNA was gel purified and sequenced in both directions on an ABI 377 sequencer.

cDNA clones. Human cDNA libraries were screened with ^{32}P labelled hybrid selected or exon trapped clones. Phage eluted from tertiary plaques were PCR amplified with vector specific primers and then sequenced on an ABI 377 sequencer.

Northern blots. Multiple tissue northern (MTN) filters, which are loaded with 2 µg per lane of poly(A)$^+$ RNA derived from a number of human tissues, were purchased from Clontech. ^{32}P-random-primer labelled probes corresponding to the 1.55 kb PCR fragment 1-Br:CG026→5 kb (exon 7/8 junction into exon 11), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to probe the filters. Prehybridizations were at 42° C in 50% formamide, 5× SSPE, 1% SDS, 5× Denhardt's mixture, 0.2 mg/ml denatured salmon testis DNA and 2 µg/ml Poly-(A)$^+$. Hybridizations were in the same solution with the addition dextran sulfate to 4% and probe. Stringency washes were in 0.1× SSC/0.1% SDS at 50 °C.

RT-PCR analysis. Poly-(A)$^+$ RNA extracted from 11 human tissues was reverse transcribed using random primes and Superscript II reverse transcriptase (Gibco BRL). Thereafter, 0.4 ng of each cDNA sample was amplified for 20 cycles using the *BRCA2* primers B2≠F9833 (5'–CGTACACTGCTCAAATCATTC) and B2≠R10061 (5'–GACTAACAGGTGGAGGTAAAG). Samples were diluted 10-fold, and then 2 µl aliquots reamplified for 18 cycles using the primers B2≠F9857 (5'–GTACAGGAAA-CAAGCTTCTGA) and B2≠R10061.

PCR amplification and mutation screening. All 26 coding exons of *BRCA2* and their associated splice sites were amplified from genomic DNA as described[17]. The DNA sequences of the primers, some of which lie in flanking intron sequence, used for amplification and sequencing appear in Table 2. Some of the exons (2 through 10, 11-5, 11-6, 11-7, and 23 through 27) were amplified by a simple one step method. The PCR conditions for those exons were: single denaturing step of 95 °C (1 min); 40 cycles of 96 °C (6 sec), $T_{ann.}$=55 °C (15 sec), 72 °C (1 min). Other exons (11-22) required nested reamplification after the primary PCR reaction. In these cases the initial amplification was carried out with the primers in the first two columns of Table 2 for 19 cycles as described above. Nested reamplification for these exons was carried out for 28 or 32 cycles at the same conditions with the primers appearing in the third column of Table 2. The products were purified from 0.8% agarose gels using Qiaex columns (Qiagen). The purified products were analysed by cycle sequencing with α-p^{32}dATP with AmpliCycle™ Sequencing Kit (Perkin Elmer, Branchburg, New Jersey). The reaction products were fractionated on 6% polyacrylamide gels. All (A) reactions were loaded adjacent to each other, followed by the (C) reactions, and so on. Detection of polymorphisms was carried out visually and confirmed on the other strand.

Acknowledgements

J.M.R. and J.S. are Scholars of the Medical Research Council of Canada. J.S. and F.L. are supported by the Medical Research Council of Canada and Endorecherche, Inc. This work was supported by NIH grants CA57601 (B.W.) and F32 CA67403 (F.C.). S.D.M. is supported by grant GCRC-MCAP-3MO1RR00042-34S1. J.E.E and S.T. are supported by the Icelandic Cancer Society Science Fund. S.N., L.C.-A., M.H.S. and D.E.G. are supported by U.S. Army grant DAMD17-94-J-4260 and NIH grants CA55914, CA48711, CA65673, RR00064, CN05222 and CA42014.

Received 11 January; accepted 6 February 1996.

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A single *BRCA2* mutation in male and female breast cancer families from Iceland with varied cancer phenotypes

Steinunn Thorlacius[1], Gudridur Olafsdottir[2], Laufey Tryggvadottir[2], Susan Neuhausen[3], Jon G. Jonasson[4], Sean V. Tavtigian[5], Hrafn Tulinius[2], Helga M. Ögmundsdottir[1] & Jorunn E. Eyfjörd[1]

The *BRCA2* gene on chromosome 13 has been shown to be associated with familial male and female breast cancer. Here we describe a study on *BRCA2* in 21 Icelandic families, including 9 with male breast cancer. We have previously reported linkage to the *BRCA2* region in an Icelandic male breast cancer family[1] and subsequently found a strong indication of linkage to *BRCA2* and the same *BRCA2* haplotype in breast cancer cases from 15 additional families, indicating a common origin. We describe a five base-pair deletion in exon 9 of *BRCA2* in an affected male from the male breast cancer family[2]. The same mutation occurs in all the families with the shared *BRCA2* haplotype indicating a founder effect. Among mutation carriers there are 12 males with breast cancer, which accounts for 40% of all males diagnosed with breast cancer in Iceland over the past 40 years. Three of them have no family history of breast cancer indicating that this mutation may have variable penetrance. The same *BRCA2* mutation appears to be associated with different cancer phenotypes in this population including male and female breast cancer, prostate cancer, pancreas cancer and ovarian cancer.

[1]*Molecular and Cell Biology Research Laboratory, Icelandic Cancer Society, P.O. Box 5420, 125 Reykjavik, Iceland*
[2]*Cancer Registry, Icelandic Cancer Society, P.O. Box 5420, 125 Reykjavik, Iceland*
[3]*Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah, USA*
[4]*Department of Pathology, University of Iceland, P.O. Box 1465, 121 Reykjavik, Iceland*
[5]*Myriad Genetics Inc., Salt Lake City, Utah, USA*

Correspondence should be addressed to J.E.E. e-mail: jorunne@ismennt.is

The *BRCA2* gene has 27 exons and encodes for a protein of 3,418 amino acids[2]. Mutations in the *BRCA2* gene are found in families with a high incidence of breast cancer and have also been shown to be involved in male breast cancer[1–3]. A five base-pair deletion, starting at nucleotide 999, codon 257 in exon 9 of the *BRCA2* gene, was detected in an affected male from an Icelandic *BRCA2* linked family with four cases of male breast cancer (Fig. 1, family 2204)[2]. Mutation analysis is in complete agreement with our previous haplotype analysis.

In the present study, we included 21 families (Table 1) selected on the basis of a high frequency of breast cancer in females or the occurrence of one or more cases of male breast cancer (see Methods). We had previously obtained strong evidence for *BRCA2* linkage with a shared haplotype in affected individuals from 16 of these families, thus suggesting a common origin (manuscript in preparation). Mutation screening revealed the 999del5 in all of these. One family (2206, Fig. 1) with a male breast cancer case does not have the *BRCA2* mutation and does not show linkage to the *BRCA2* region. The other families negative for 999del5 have also been excluded for *BRCA2* linkage based on haplotype analysis. We have not found linkage to *BRCA1* in these families. Male breast cancer occurred in 9 of the 16 families positive for the *BRCA2* mutation (12 individuals). A total of 30 males have been diagnosed with breast cancer in Iceland over the last 40 years[4] and these have all been screened for 999del5. The same mutation and the associated haplotype was found in 12 of these cases (40%). The 999del5 is a frameshift mutation leading to an early termination in codon 273 and a highly truncated protein. Loss of the wild-type *BRCA2* allele was detected in all the tumours tested (Fig. 2). It is therefore clear that there is a total loss of functional *BRCA2* protein in the tumours of mutation carriers.

The breast cancer incidence in Iceland is similar to that in other western countries, with male breast cancers representing about 1% of breast carcinomas. There is no evidence that familial breast cancer is more frequent in Iceland than elsewhere[5]. The high frequency of the same *BRCA2* mutation in the Icelandic breast cancer families indicates a founder effect. This is supported by the finding of the common haplotype in mutation carriers. This allows us to speculate on the age of the mutation in the population. At least six of the 16 families can be traced back to a common ancestor in the middle of the 16th century (11–12 generations). High frequency of a specific *BRCA1* mutation has been reported in individuals of Ashkenazi Jewish origin, indicating a small founding population[6]. It will be of interest to determine the prevalence of the 999del5 mutation in the Icelandic population. We are currently screening over 500 sporadic female breast cancer patients and 500 controls. So far *BRCA1* mutation has only been identified in one Icelandic family[7], indicating that it might be more recent in the population than the mutation in the *BRCA2* gene.

The mean age of onset for female breast cancer varies between the mutation positive families (35.5–72.0). Forty-four out of 100 female breast cancer cases in the mutation positive families have been tested. Thirty-five of these have the 999del5 (79.5%) which is in agreement with the haplotype analysis. The nine cases without the mutation, likely to be sporadic, have a mean age of onset of 61.5 years as compared to 50.6 years for the mutation positive cases. Mutation screening has not been completed in all cancer cases in the mutation positive families but the 999del5 has been found in individuals with different tumour types including cancer of the prostate, pancreas, ovary, colon, stomach, thyroid, cervix and endometrium. In the mutation positive families there are 26 cases of prostate cancer. The four prostate cancer cases in the male breast cancer family 2204 all tested negative for the mutation. In three other *BRCA2* families (2201, 2211 and 2212) all prostate cancers cases tested so far are mutation carriers. No male breast cancers have occurred in these families. The same mutation is therefore clearly associated with male breast cancer in some families but not in others, suggesting modification by other genes and/or environmental factors that may affect the phenotypic expression. In the mutation positive families there are 11 pancreatic cancers but none were found in the negative families (Table 1*a*, *b*). Information on mutation status is available for two of these and both are positive for the mutation. This is of

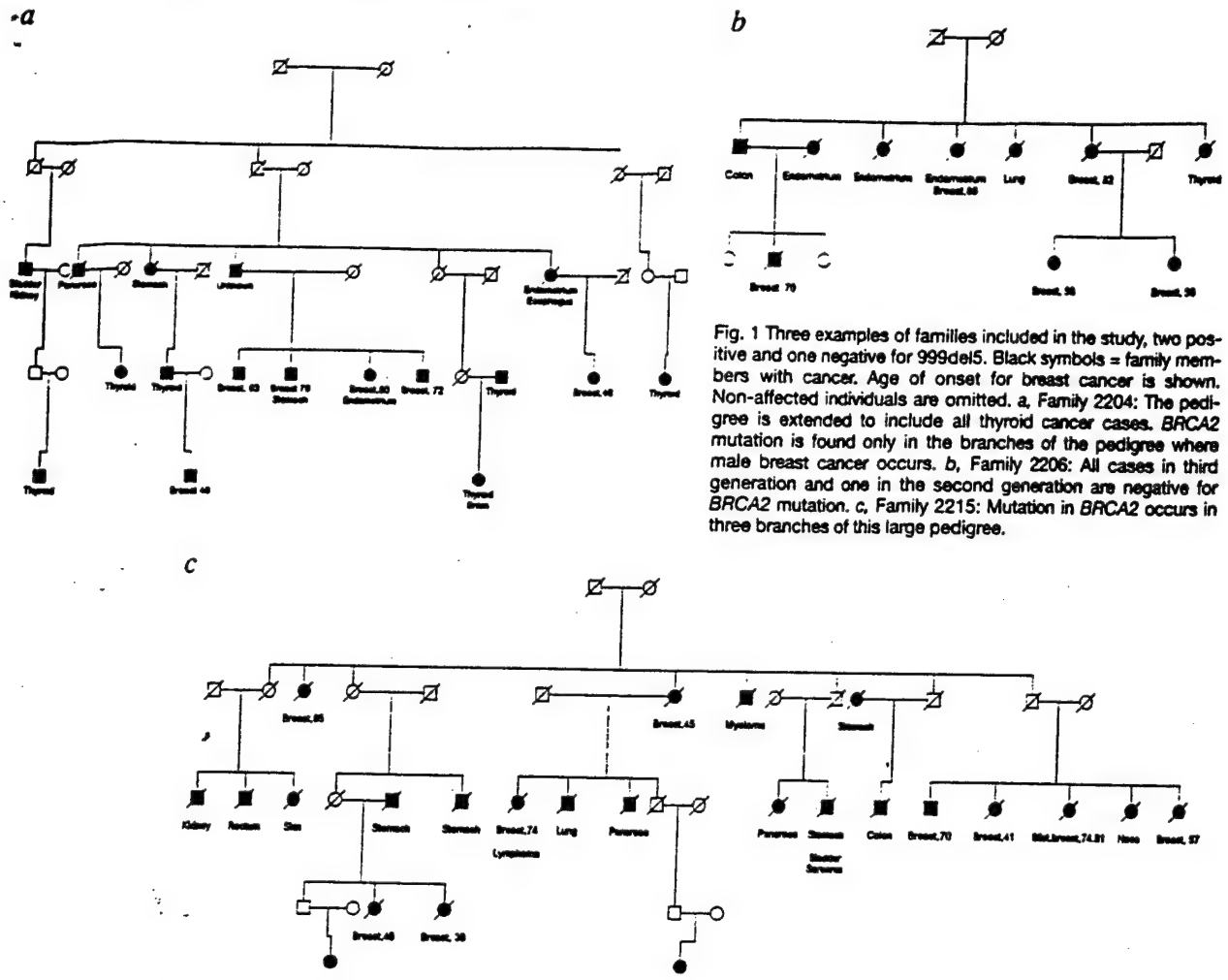

Fig. 1 Three examples of families included in the study, two positive and one negative for 999del5. Black symbols = family members with cancer. Age of onset for breast cancer is shown. Non-affected individuals are omitted. *a*, Family 2204: The pedigree is extended to include all thyroid cancer cases. *BRCA2* mutation is found only in the branches of the pedigree where male breast cancer occurs. *b*, Family 2206: All cases in third generation and one in the second generation are negative for *BRCA2* mutation. *c*, Family 2215: Mutation in *BRCA2* occurs in three branches of this large pedigree.

interest since a homozygous deletion found in a pancreatic cancer helped in localizing the *BRCA2* gene[8]. In an extended pedigree of family 2204 five cases of thyroid cancer have occurred (Fig. 1). One thyroid cancer case has the *BRCA2* germline mutation whereas four have neither the haplotype nor the mutation.

In one family, parents carried the same *BRCA2* mutation but no 999del5 homozygotes were found among their offspring. Similar findings (a couple with obligate identical haplotype) were reported in a large *BRCA2* linked French–Canadian family[9]. This raises the question of viability of *BRCA2* homozygotes. The three male breast cancer patients (families 2219, 2220 and 2221), positive for 999del5, with little or no family history of breast cancer imply that penetrance in *BRCA2* heterozygotes may vary between families. Most reports on *BRCA2* to date have been based on highly selected families with high penetrance. According to our study there appear to be a number of unaffected mutation carriers (obligate carriers, unaffected, above 70 years-of-age) indicating that penetrance may be quite low in some families.

The identification of the *BRCA2* gene was a significant step towards understanding familial breast cancer. The material presented here clearly shows that the same mutation can be associated with different cancer phenotypes and variable expression, emphasizing the need for identifying potential modifying factors, genetic as well as environmental. This will be of vital importance for future genetic counselling.

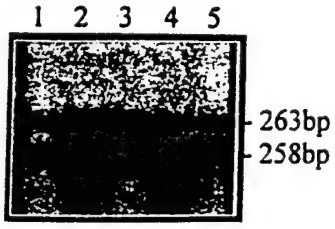

Fig. 2 Detection of 999del5 by PCR amplification and PAGE. Exon 9 of the *BRCA2* gene was amplified and run on a 6% acrylamide denaturing gel. The normal PCR product is 263 bp but the mutated one is 258 bp. Lane 1, 2 and 5; DNA from blood samples from individuals heterozygous for 999del5. Lane 4; Tumour DNA from the same individual as in lane 5, with almost complete loss of the wild-type allele. Lane 3; normal control.

Methods

Families. The Icelandic Cancer Society from 1986 has collected samples from individuals in high risk cancer families, as well as samples from the majority of all breast cancer patients diagnosed in Iceland over this period. Information on family history of cancer was obtained from the Family Resource of the Icelandic Cancer Registry. From our collection we selected twenty-one families for *BRCA2* analysis using the following criteria: i) three or more first degree relatives with breast cancer and/or ii) cases occurring in at least three generations or iii) one or more cases of male breast cancer. Male breast cancer occurred in nine of our families. We obtained samples from all male breast cancer cases diagnosed in Iceland 1954–1995 — a total of 30 cases — from the Department of Pathology, University Hospital Iceland. DNA was isolated from whole blood, lymphocytes or fresh tissue by standard phenol/chloroform extraction. DNA from paraffin embedded material was extracted as described[10].

Table 1 Cancer sites in 21 families tested for 999del5

			Breast			Other sites, both sexes														
a,	No. of family members																			
Families	F	M	F	<55	≥55	M	Co	Pa	Ga	Rs	Ov	Ge	Pr	Ur	Sk	Br	Th	Sa	Re	Ot	Total
2201	190	174	12	10	2		1	1	1	2	3	1	4		1	1	2		3	3	35
2202	126	157	19	11	8	1	4		3	3	1			2			1			1	35
2203	24	16	3	2	1	1	1	1			1		1						1		9
2204	81	88	2	1	1	4		1	4	1	1	3	3	1		1	2	2		2	27
2207	30	44	7	4	3			1	1		1		1							1	12
2209	63	60	9	7	2		2			2	2	1		4			1				21
2211	35	39	9	5	4			2					4								15
2212	61	61	10	7	3				2		1		5	2							20
2214	24	25	5	5			1						2								8
2215	167	172	11	6	5	1	2	2	2	3					3	2	1	2	3		32
2216	32	40	8	1	7		2						1		1		1				13
2217	37	34	2	2		1														1	4
2218	37	37	2		2	1		3	3	1	2	1	1								14
2219	377	427	0			1	3		4	4			2			1			3	1	19
2220	70	88	1	1		1				1			1					1	1	1	7
2221	60	67	0			1	1		1				1			2					6
Total	1392	1513	100			12	17	11	21	17	12	6	26	9	5	7	8	5	11	10	276
b,																					
2205	42	37	5	4	1				2	1	1									1	10
2206	261	251	6	1	5	1	2		1	2						2			1	1	16
2208	66	44	7	6	1		1		1		1						2				12
2210	209	228	8	4	4		2		2	1			2	3		1				1	20
2213	78	83	8	2	6					1				1	1						11
Total	656	643	34			1	5		6	5	2		2	4	1	3	2		1	3	69

The tables lists all cancers in first and second degree relatives and at least all third degree relatives with cancer in the same generation as the proband. *a,* Families with the 999del5 mutation. *b,* Families without the 999del5. F = female breast cancer; M = male breast cancer; <55 = female breast cancer diagnosed under age 55; ≥55 = female breast cancer diagnosed at age 55 or older. Co = colon/rectal cancer, Pa = pancreas cancer, Ga = other gastrointestinal cancers, Rs = cancers of the respiratory tract, Ov = ovarian cancer, Ge = other genital cancers, Pr = prostate cancer, Ur = urinary cancer, Sk = skin cancer, Br = brain cancer, Th

Haplotype analysis and mutation screening. Polymorphic microsatellite markers flanking the *BRCA2* locus were used for haplotype analysis. Exon 9 of the *BRCA2* gene was amplified using primers as published[2]. DNA was amplified by PCR using α-P^{32}CTP labelling, the product was run on 6% denaturant PAGE and made visible by autoradiography. Suspected *BRCA2* mutants were identified as having an extra band, smaller than the normal PCR fragment.

DNA sequencing. One or more samples from each family were sequenced to verify the mutation. Solid phase sequencing was carried out using the same primers as in the mutation screening, except the forward primer was 5' biotinylated. The biotinylated PCR product was bound to streptavidin coated Dynabeads M280 (Dynal, Norway) and sequenced as described[11]. Linkage analysis was performed using the LINKAGE program[12].

Acknowledgements

We thank the family members for their contribution; J. Hallgrimsson and staff at the Department of Pathology for tissue samples from tumours and from deceased individuals; the Genetical Committee of the University of Iceland for genealogical information; and D. Goldgar, University of Utah/ IARC France, for helpful discussion and for primers. This work was supported by grants from the Icelandic Cancer Society Science Fund. S.N. was supported by grant DAMD17-94-J-4260. J.E.E. is a member of EU Breast Cancer Linkage Consortium.

Received 29 February; accepted 25 March 1996.

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Frequency of recurrent *BRCA1* and *BRCA2* mutations in Ashkenazi Jewish breast cancer families

Two founding mutations in *BRCA1* and *BRCA2* are responsible for a large proportion of Jewish families with the breast-ovarian cancer syndrome.

P. TONIN[1], B. WEBER[2], K. OFFIT[3], F. COUCH[2], T.R. REBBECK[4], S. NEUHAUSEN[5], A.K. GODWIN[6], M. DALY[7], J. WAGNER-COSTALOS[7], D. BERMAN[8], G. GRANA[8], E. FOX[9], M.F. KANE[9], R.D. KOLODNER[9], M. KRAINER[10], D.A. HABER[10], J.P. STRUEWING[11], E. WARNER[12], B. ROSEN[13], C. LERMAN[14], B. PESHKIN[14], L. NORTON[15], O. SEROVA[16], W.D. FOULKES[17], H.T. LYNCH[18], G.M. LENOIR[16], S.A. NAROD[19] & J.E. GARBER[20]

Germline mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* account for the majority of families with hereditary breast cancer. Among women with *BRCA1* mutations, the lifetime risk of breast cancer exceeds 80%, and the risk of ovarian cancer approaches 50% (ref. 1). The risk for breast cancer in carriers of *BRCA2* mutations is also high, but may vary with the position or type of mutation. Although the risk of ovarian cancer is lower for *BRCA2* than for *BRCA1* mutation carriers, several other cancers appear to be part of the *BRCA2* spectrum, including male breast cancer and pancreatic cancer[2-4].

Both genes are large: the coding region of *BRCA1* contains 5,592 base pairs distributed over 22 exons and *BRCA2* contains 10,254 base pairs of coding sequence within 27 exons[4,5]. Because of the size of these genes, mutation detection is difficult by current techniques. The task of ruling out a mutation in both genes represents a considerable undertaking for the diagnostic laboratory — although this is what the patient or physician most often requests. Because of the similarity in the sites of cancer that are associated with the two genes, exclusion of a mutation in a single gene is of limited clinical value.

Both unique and recurrent mutations in *BRCA1* and *BRCA2* have been described; the majority are small deletions and insertions that lead to premature protein truncation. Over 100 different *BRCA1* mutations have been reported worldwide[7], but the number of different mutations present in a defined ethnic group may be limited. In particular, four distinct recurrent mutations (see Table 1) have been found in the Ashkenazi Jewish population (the Ashkenazim are Jews presently or formerly from Eastern Europe)[4,8-12]. The most common of these mutations appears to be the deletion of thymidine at position 6174 (6174delT), a *BRCA2* mutation, which was been reported in 1.4% of unselected Ashkenazi Jews[12-14]. The most common *BRCA1* mutation, 185 delAG, is estimated to be present in approximately 1% of the Jewish population[14,15], or roughly eight times that of all *BRCA1* mutations combined in the general population[14]. The cytosine insertion at position 5382 (5382insC) *BRCA1* mutation has been seen in both Jewish and non-Jewish breast cancer families and is present in approximately 0.1% of the Jewish population[14]. Recently, a third *BRCA1* mutation (188del11) was reported in four Ashkenazi Jewish women with breast or ovarian cancer[11]. This mutation was not seen in a survey of 3000 unselected Jews[11].

These studies together imply that the frequency of breast cancer susceptibility mutations in the Jewish population probably exceeds 2%. Based on this prevalence, we predict a high frequency of *BRCA1* and *BRCA2* mutations among Jewish families with breast cancer and that the lifetime cancer risk associated with a family history of breast cancer might be greater for Jewish than for non-Jewish women[17]. Although there have been several reports of recurrent *BRCA1* and *BRCA2* mutations in Jewish women with breast cancer[4,8-12,18], no study has screened the subjects for all four mutations or has been sufficiently large to provide precise figures for clinical use. To estimate the proportion of breast cancer families attributable to these four mutations, we have screened a panel of 220 North American Jewish breast cancer families. Each family contained two or more cases of breast cancer, and a family member presented for testing in research protocols conducted at Memorial Sloan-Kettering Hospital (67 families), the Dana-Farber Cancer Center (38 families), McGill University (27 families), the Fox Chase Cancer Center (29 families), the University of Pennsylvania (26 families), the Massachusetts General Hospital (13 families), the National Institutes of Health (7 families), Creighton University (6 families), the University of Toronto (5 families), and the Lombardi Cancer Center (2 families).

The families

Each family contained a minimum of two women affected with breast cancer (mean 3.5 cases, range 2–11 cases), at least one of whom was diagnosed with breast cancer at age 50 or below. Families with cases of male breast cancer were excluded. Among the 220 eligible families, 82 also contained cases of ovarian cancer. At least one affected individual in each family was screened for the 185delAG, 188del11 and the 5382insC *BRCA1* mutations and for the 6174delT *BRCA2* mutation. Where possible, the screened individual was the youngest

Table 1 The four mutations screened for

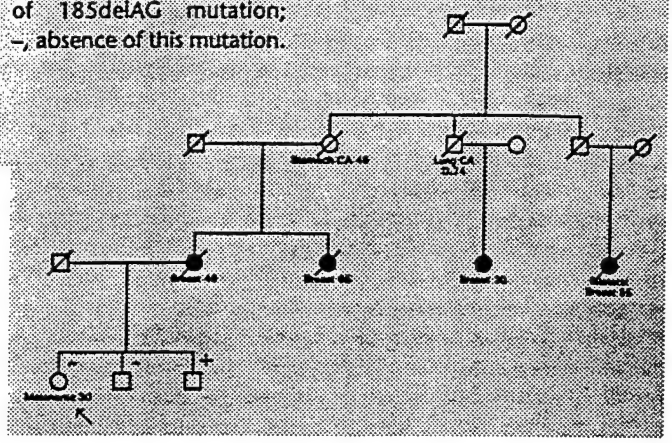
Gene	Mutation	Exon	Frequency in Jewish population	References
BRCA1	185delAG	2	1.05%	14, 15
	188del11	2	0.0%	14
	5382insC	20	0.11%	14, 15
BRCA2	6174delT	11	1.36%	13, 14

The frequency figures are based on a combined analysis of the data in the cited references.

Box 1 Mutation detection in the absence of a living affected individual

A common problem in genetic testing is the availability of DNA material from an affected index case for mutation testing. The identification of frequent recurrent mutations opens the possibility for genetic testing of asymptomatic women in the absence of an index case. There are four cases of breast cancer in this family, two below age 50, but none of these individuals were available for testing. The proband (↑) was diagnosed with malignant melanoma at age 30, and wished to be tested for breast cancer susceptibility. Blood was available from her and her two brothers, and DNA screening for the three mutations was offered. The 185delAG mutation was found in the proband's brother, but not in the proband, and she is considered not to be at increased risk for breast cancer. The effectiveness of this approach depends on the willingness of family members to share results with each other. Of course, disclosure of test results to other family members is not routine, nor is the agreement to do so a prerequisite for genetic counseling, but in our experience this degree of cooperation is not unusual.

●, Women with breast or ovarian cancer. Ages of diagnosis of cancers are given below symbols. /, Deceased; +, presence of 185delAG mutation; –, absence of this mutation.



living case of breast cancer in the family for whom DNA was available. In a small number of cases, a woman with ovarian cancer or an obligate gene carrier was screened. Mutation detection was accomplished by direct sequencing, sequence-specific conformation analysis (SSCA), heteroduplex analysis, allele-specific PCR or allele-specific oligonucleotide (ASO) analysis as previously described[4,9,10,12,13,14,16,20].

Frequency of recurrent *BRCA1* and *BRCA2* mutations

At least one woman with breast or ovarian cancer from each family was screened for the four mutations. A total of 100 families (45.5%) carried *BRCA1* or *BRCA2* mutations, including 71 families with the 185delAG mutation (32.3%), 20 families with 5382insC mutation (9.1%) and 9 families with the 6174delT mutation (4.1%) (Table 2). The 5382insC mutation was the second most common mutation in both the site-specific breast cancer and the breast-ovarian cancer subgroups and accounted for more than 20% of the 91 *BRCA1* mutations in this series. The 188del11 mutation was not found in any of the 220 individuals tested.

Penetrance of the mutations

The frequency of the 6174delT mutation is estimated to be 1.4% in the Jewish population[13,14]. This mutation was previously reported to be present in 8% of Jewish women with breast cancer diagnosed before the age of 42 (ref. 12) and in 3% of cases diagnosed between ages 42 and 80 (1 out of 29)(ref. 13). It is surprising that this common mutation was seen in only 4% of the breast cancer families in the present data set. This is in keeping with the conclusions of others that the penetrance of the 6174delT mutation is much lower than that of the *BRCA1* mutations; Roa and colleagues estimate the lifetime risk to be less than 25% (ref. 14). The 5382insC mutation is approximately one-tenth as frequent as either the 6174delT mutation or the 185delAG mutation, but it accounted for 22% of our *BRCA1* mutated families. This observation suggests that the penetrance of 5382insC may exceed that of 185delAG or that the frequency of the 5382insC mutation may have been underestimated.

Several groups have claimed that the risk of ovarian cancer appears to be less for individuals with 3′ *BRCA1* mutations compared with carriers of mutations that are positioned more 5′ in the gene[21–23]. We were able to test this hypothesis by comparing the average numbers of cancers in the 71 families with the 5′ mutation, 185delAG, with the 20 families with the 3′ mutation, 5382insC. The average number of cases of ovarian cancer was slightly higher in families with the 185delAG mutation (1.25 cases) than with the 5382insC mutation (1.00 cases),

Table 2 Frequency of *BRCA1* and *BRCA2* mutations in Jewish breast cancer families

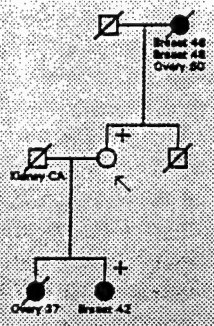
Site-specific breast cancer (no ovarian cancer)

		Mutation (%)			
	Total families	185delAG	5382insC	6174delT	Any (%)
2 breast cancers	48	10	2	0	12 (25.0%)
3 breast cancers	43	7	3	1	11 (25.6%)
4+ breast cancers	47	11	2	4	17 (36.1%)
Total	138	28	7	5	40 (29.0%)

Breast-ovarian cancer syndrome

		Mutation (%)			
	Total families	185delAG	5382insC	6174delT	Any (%)
2+ breast, 1 ovarian	54	22	9	4	35 (64.8%)
2+ breast, 2+ ovarian	28	21	4	0	25 (89.3%)
Total	82	43	13	4	60 (73.2%)

Box 2 The importance of oophorectomy



If we assume a genetic basis for cancer exists in this family, the mother (↑), although alive and unaffected at age 68, appears to be an obligate carrier. The 185delAG mutation was found in her and in her living daughter. Although she is an example of a nonpenetrant carrier of the 185delAG mutation, she had undergone a bilateral oophorectomy in her forties. We recommend that oophorectomy be recorded for all at-risk women in breast cancer families. It is also important that such surgeries be taken into account in epidemiologic studies when penetrance estimates of mutations are constructed from family data.

However, this difference was not statistically significant (P = 0.78). We do not feel that the issue of mutation-specific penetrances is resolved for *BRCA1*, and therefore it is not yet appropriate to provide mutation-specific risk estimates in Jewish women.

Age of breast cancer diagnosis and presence of mutation

The average number of cases of breast cancer in families with *BRCA1* mutations (3.58 cases) was similar to that of families with no mutation detected (3.41 cases) and to that of families with *BRCA2* mutations (3.89 cases). The average age of breast cancer diagnosis among the families with a *BRCA1* mutation was 45.1 years. This was slightly younger than the average age of 47.1 years for the 120 families with no mutation detected, and slightly older than the mean age of 42.2 years for the nine families with *BRCA2* mutations. There were four families that contained at least one unusually young case of breast cancer (<25 years); it is surprising that none of these contained mutations. Among these four families, two families reported cases of colon cancer, two families prostate cancer, and one a case of leukemia. It is possible that these families with very young cases represent a distinct genetic entity or have different *BRCA1* or *BRCA2* mutations.

The importance of ovarian cancer

A family history of ovarian cancer was a strong risk factor for the presence of a *BRCA1* mutation in this family set [odds ratio (OR) = 6.7; $P < 0.0001$]. Sixty percent of families with mutations contained at least one case of ovarian cancer. Overall, mutations were found in 29.0% of families with no case of ovarian cancer, in 64.8% of families with one ovarian cancer, and in 89.3% of families with two or more ovarian cancers (Table 2). The latter figure is consistent with the previous estimate that 90% of families with two or more cases of ovarian cancer and two or more cases of early-onset breast cancer carry a *BRCA1* mutation[24].

Table 3 The proportion of families with mutations associated with the presence of cancers at other sites

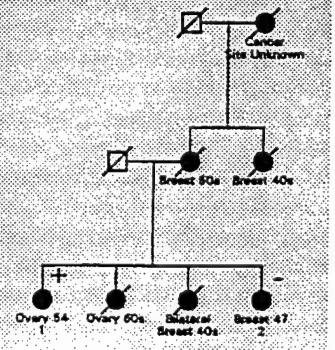
	Number of families	Percent with mutations	Relative risk
Colon	53	54.7	1.63
Prostate	28	39.3	0.75
Stomach	24	37.5	0.70
Leukemia	19	42.1	0.86
Pancreas	19	73.7	3.73*
Lymphoma	18	44.4	0.80
Brain	15	33.3	0.58
Skin	14	35.7	0.65
Endometrial	10	60.0	1.85
Melanoma	8	50.0	1.21
Kidney	8	25.0	0.39
ENT	6	33.3	0.59
Hodgkins	6	50.0	1.20
Liver	6	33.3	0.59
Bladder	6	16.7	0.23
Osteosarcoma	6	50.0	1.20
Esophagus	5	60.0	1.82
Cervix	5	50.0	1.82
Fallopian	4	100	inf*
Biliary	2	100	inf
Eye	2	100	inf
Thyroid	2	50.0	1.20
Testes	1	0.00	0.00
Omentum	1	100	inf

Abbreviation: inf, odds ratio infinite. *$P < 0.05$

Predictive value of cancer at other sites

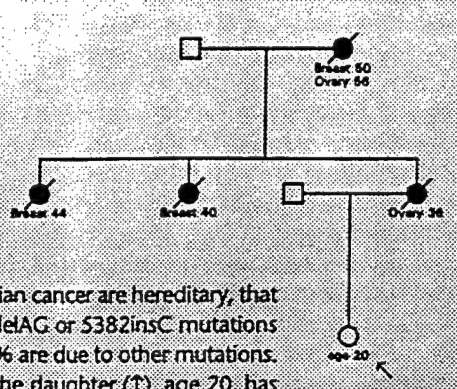
The proband was questioned about her family history of cancers at all sites. In general, cancer at these other sites was not confirmed by pathology report, and the mutation status of these affected relatives is unknown. A family history of pancreatic cancer was predictive of the presence of a mutation in the breast cancer families (Table 3). Fourteen of the 19 families (73.7%) with pancreatic cancer were found to have a mutation (11 in *BRCA1* and three in *BRCA2*); compared with 42.8% of families without pancreatic cancer (OR = 3.74; P = 0.01). A family history of pancreatic cancer was a particularly strong predictor of having a *BRCA2* mutation (OR = 6.1; P =

Box 3 The problem of sporadic cases



Sporadic (nonhereditary) breast cancer cases and hereditary cases may be found in the same family. It is estimated that 90% of families with two or more cases of breast cancer and two or more cases of ovarian cancer carry one of the two *BRCA1* mutations (Table 2). Before mutation analysis, a limited linkage analysis was done on this family. Affected individuals 1 and 2 did not share a common chromosome 17q haplotype for markers at the *BRCA1* locus. However, because of the very high prior probability of a *BRCA1* mutation in this family, both individuals were screened for the recurrent mutations, and a 185delAG mutation was found in individual 1. Individual 2 was negative for the four mutations. It is not yet known which additional cases in this family are attributable to the 185delAG mutation, but this case illustrates the importance of considering the entire family in genetic risk assessment.

Box 4 The interpretation of a negative result



Because of the high proportion of mutations that are accounted for by three variants in Jewish families, a negative mutation test will have predictive value in some families with the breast-ovarian cancer syndrome. For this risk assessment we assume that all families with two or more cases of early-onset breast cancer and two or more cases of ovarian cancer are hereditary, that 90% of these are due to either 185delAG or 5382insC mutations (Table 2), and that the remaining 10% are due to other mutations. *A priori*, there is a 50% chance that the daughter (↑), age 20, has inherited a normal chromosome 17, a 45% chance that she has inherited either a 185delAG or a 5382insC mutation, and a 5% chance that she has inherited a different *BRCA1* or *BRCA2* mutation. If no living affected relative were available for testing, and the proband were found to be negative for the two specified mutations, then only the first and last possibilities would remain (with relative probabilities of 50% and 5%). Her risk of carrying a *BRCA1* mutation would therefore fall from 50% to 9.1% (that is, 50/(50+5)). In order to reduce the risk further, it would be necessary to look for other mutations by a comprehensive screening approach.

0.03). An excess of pancreatic cancer has been reported in families with *BRCA2* mutations in Canada and in Iceland[4,25]. Another risk factor appears to be a history of cancer of the fallopian tube. All four families with fallopian tube cancer carried mutations, three had a 185delAG mutation and one had a 6174delT mutation. This frequency was significantly greater than expected ($P = 0.04$). This association is intriguing, given the pathological similarity of fallopian and ovarian cancers, but will need to be confirmed in a larger data set.

Genetic counseling of Ashkenazi breast cancer families

The observation of recurrent *BRCA1* and *BRCA2* mutations in a defined subgroup of the population may lead to a cost-effective method for mutation screening. Therefore, we sought to estimate precisely the proportion of familial breast cancers in the Jewish population attributable to each of these four mutations. This knowledge is critical for formulating policies for genetic screening within this ethnic group and for accurately interpreting genetic test data in making clinical decisions. By agreeing on common testing criteria and by combining the results generated in our nine centers we were able to address this issue. We believe that the process of genetic testing and risk interpretation should take into account the high frequency of a limited number of mutations in Ashkenazi Jewish women.

We propose that genetic screening of Ashkenazi Jewish women at risk for breast and ovarian cancer consist of, as a minimum, a method to detect these three mutations. It is possible that there will be other *BRCA1* or *BRCA2* mutations identified in Ashkenazi Jews, but these will likely be less frequent than those reported here. Some centers may wish to pursue additional testing on Jewish families who do not show evidence for any of these three mutations.

Our families were selected for the presence of two or more cases of breast cancer, or a single case of breast cancer at age 50 or below. These inclusion criteria are less stringent than the accepted definition of a hereditary breast cancer family (for example, four or more cases of breast or ovarian cancer). We identified mutations in 25% of families with only two cases of breast cancer (and no case of ovarian cancer). The sensitivity of using this panel of three mutations cannot easily be assessed on small families, because it is likely that most small families lack mutations. However, the findings presented here imply that if testing is to be offered, such small families should be eligible. We feel that where expertise is available, including genetic counseling and suitable follow-up, *BRCA1* and *BRCA2* mutation detection is an appropriate clinical test for Jewish women at high risk of cancer.

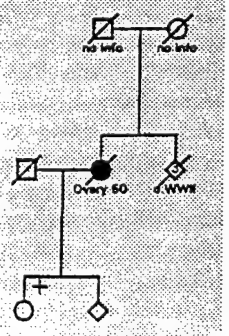
Studies are now under way to estimate the frequency of *BRCA1* and *BRCA2* mutations in unselected Jewish women with breast or ovarian cancer and in the general Jewish population. The results of these studies will be important in determining whether or not all Jewish women with cancer should be candidates for genetic testing in the absence of a family history of breast or ovarian cancer, and for interpreting both positive and negative tests in these small families.

Acknowledgments

We thank Susan Hew, Steven Lemon, Carrie Snyder, Carolyn Durham, Corinne Serruya, Deborah MacDonald, Roxana Moslehi, David Cole and Elizabeth Hoodfar for their assistance with family collection, and Paul Morrison, Christine Earabino, Maria Galvez, Marie-Claude Faucher, Kirin Dole, David Rosenblatt, Christine Larkin and Jean-Sebastien Brunet for technical assistance.

This work was supported by National Institutes of Health grants CA 06516, AI 28691, CA 55914, CA 606643 and CA 57601; US Army grants DAMD17-94-J-4299, DAMD17-94-J-H260, DAMD17-94-J-4425, the Friends of the Dana-Farber Cancer Institute, the Charles A. Dana Foundation, the Cancer Research Society, Inc., the Canadian Breast Cancer Foundation

Box 5 How strong a family history is needed for testing to proceed?



Given the frequency of *BRCA1* and *BRCA2* mutations in Ashkenazim, the question arises: should all Jewish women with breast or ovarian cancer, or a family history of cancer, be offered screening? This figure illustrates the case of an Ashkenazi woman who requested testing because of a single affected relative. Her mother was diagnosed with serous papillary adenocarcinoma at age 50. The family history was limited, and several relatives had perished at a young age in the Holocaust. Because the possibility of hereditary cancer could not be excluded with her family history, she was offered predictive testing and a 185delAG mutation was identified.

(Ontario Chapter), the Canadian Genetics Diseases Network, the Fonds de Recherche en Santé du Quebec, the Emile Zola Chapter of Brith Shalom Women and the Komen Foundation. P.T. is a scholar of the Medical Research Council of Canada and the Cancer Research Society of Canada.

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[1]*Department of Medicine, Division of Medical Genetics, and Department of Human Genetics*
McGill University and Montreal General Research Institute
1650 Cedar Avenue, Montreal, Canada, H3G 1A4
[2]*Departments of Medicine and Genetics, University of Pennsylvania*
3400 Spruce Street, Philadelphia, Pennsylvania 19104, USA
[3]*Clinical Genetics Service, Department of Human Genetics*
Memorial Sloan-Kettering Cancer Center, 1275 York Avenue
New York, New York 10021, USA
[4]*Department of Biostatistics and Epidemiology*
University of Pennsylvania, 423 Guardian Drive
Philadelphia, Pennsylvania 19104, USA
[5]*Genetic Epidemiology Group, Department of Medical Informatics*
University of Utah, 391 Chipeta Way, Salt Lake City, Utah 84108, USA
[6]*Divisions of Basic Science and*
[7]*Population Science, Fox Chase Cancer Center*
7701 Burnholme Avenue, Philadelphia, Pennsylvania 19111, USA
[8]*Department of Medicine, Division of Hematology/Oncology*
Cooper Hospital, 1 Cooper Plaza, Camden, New Jersey 08103, USA
[9]*Division of Human Cancer Genetics, and*
[10]*Division of Cancer Epidemiology and Control*
Dana-Farber Cancer Institute
44 Binney Street, Boston, Massachusetts 02115, USA
[11]*Center for Cancer Risk Analysis*
Massachusetts General Hospital Cancer Center
Charlestown, Massachusetts 02129, USA
[12]*Genetic Epidemiology Branch*
National Cancer Institute and Laboratory of Gene Transfer
National Center for Human Genome Research
National Institutes of Health, 6310 Executive Boulevard
Bethesda, Maryland 20892, USA
[13]*Division of Medical Oncology*
Toronto-Sunnybrook Regional Cancer Center
2075 Bayview Avenue, Toronto, Canada M4N 3M5
[14]*Department of Obstetrics and Gynecology, The Toronto Hospital*
200 Elizabeth Street, Toronto, Canada M5G 2C4
[15]*Lombardi Cancer Center, Georgetown University Medical Center*
2233 Wisconsin Avenue, Washington, DC 20007 USA
[16]*Breast Cancer Medicine Service, Department of Medicine*
Memorial Sloan-Kettering Cancer Center, 1275 York Avenue
New York, New York 10021, USA
[17]*International Agency for Research on Cancer*
150 Cours Albert Thomas, 69372 Lyon, France
[18]*Cancer Prevention Research Unit*
Sir M.B. Davis Jewish General Hospital
Montreal, Quebec, Canada H3T 1E2
[19]*Department of Preventive Medicine and Public Health*
Creighton University School of Medicine
Omaha, Nebraska 68178, USA
[20]*Department of Medicine, Women's College Hospital*
University of Toronto, 790 Bay Street, Room 750
Toronto, Ontario, Canada M5G 1N8
[10]*Division of Cancer Epidemiology and Control*
Dana-Farber Cancer Institute, 44 Binney Street
Boston, Massachusetts 02115, USA
Correspondence should be addressed to S.A.N.

Am. J. Hum. Genet. 61:120–128, 1997

Cancer Risks in Two Large Breast Cancer Families Linked to BRCA2 on Chromosome 13q12-13

D. F. Easton,[1] L. Steele,[2] P. Fields,[2] W. Ormiston,[3] D. Averill,[4] P. A. Daly,[3] R. McManus,[3] S. L. Neuhausen,[2] D. Ford,[4] R. Wooster,[5] L. A. Cannon-Albright,[2] M. R. Stratton,[5] and D. E. Goldgar[2,*]

[1]CRC Genetic Epidemiology Unit, Institute of Public Health, Cambridge; [2]Genetic Epidemiology, Department of Medical Informatics, University of Utah, Salt Lake City; [3]Department of Medicine, Trinity College Medical School, Dublin; and Sections of [4]Epidemiology and [5]Molecular Carcinogenesis, Institute of Cancer Research, Belmont, Surrey

Summary

The penetrance of the BRCA2 gene on chromosome 13q12-13 has been estimated in two large, systematically ascertained, linked families, by use of a maximum-likelihood method to incorporate both cancer-incidence data and 13q marker typings in the families. The cumulative risk of breast cancer in female gene carriers was estimated to be 59.8% by age 50 years (95% confidence interval [95%CI] 25.9%–78.5%) and 79.5% by age 70 years (95%CI 28.9%–97.5%). The cumulative risk of breast cancer in male carriers was estimated to be 6.3% (95%CI 1.4%–25.6%) by age 70 years. There was no evidence of any risk difference between the two families. These results indicate that the lifetime breast cancer risk in BRCA2 carriers, for at least a subset of mutations, is comparable to that for BRCA1. A significant excess of ovarian cancer in gene carriers was observed (relative risk 17.69, based on three cases), but the absolute risk of ovarian cancer was less than that reported for BRCA1. Significant excesses of laryngeal cancer (relative risk 7.67, based on two possible carriers) and prostate cancer (relative risk 2.89, based on five possible carriers) were also observed. One case of ocular melanoma, as well as a second eye cancer of unspecified histology, occurred in obligate gene carriers.

Introduction

There has recently been major progress toward the identification of genes responsible for inherited predisposition to breast cancer. In a substantial proportion of families with a high risk of breast cancer, the disease is known to be due to the BRCA1 gene on chromosome 17q (Miki et al. 1994). This gene appears to account for the majority of families segregating early-onset breast and ovarian cancer, but only a minority of families segregating breast cancer only (Easton et al. 1993). Wooster et al. (1994) have demonstrated that a large proportion of such families in which the disease is not due to BRCA1 are linked to another gene on chromosome 13q12-13, known as "BRCA2," which has also now been cloned (Wooster et al. 1995). In order to provide counseling for individuals in BRCA2-linked families, it is important to be able to determine the age-specific risks of cancer associated with BRCA2 mutations. It is not usually straightforward to estimate these risks on the basis of families collected for linkage analysis, because the basis on which the families have been ascertained is usually not well determined. Although there are methods for estimating penetrance that are free from ascertainment assumptions (Risch et al. 1984; Easton et al. 1993), these methods require conditioning on all available phenotypic information and therefore provide imprecise estimates unless a large number of families are available. Fortunately, in the case of BRCA2, two large linked families have been identified that were ascertained on the basis of an initial cluster of cases and then were extended systematically. We have therefore used data on these families to estimate the age-specific risks of breast cancer in BRCA2-mutation carriers, making use of the data both on incident cancers and on 13q marker typings. We have also examined the risks of cancers, other than breast cancer, in BRCA2-mutation carriers.

Subjects and Methods

Families

Kindred UTAH107 was initially ascertained by Eldon Gardner in 1947. The family was reported to Gardner by a genetics student at the University of Utah who had two great aunts both of whom died of breast cancer in their 40s. Subsequent family tracing identified seven additional cases of breast cancer, as well as several indi-

Received May 4, 1995; accepted for publication May 1, 1997.

Address for correspondence and reprints: Dr. Douglas Easton, Department of Community Medicine, Institute of Public Health, University of Cambridge, Robinson Way, Cambridge CB2 2SR, United Kingdom. E-mail: douglas.easton@mrc-bsu.cam.ac.uk

* Present address: Unit of Genetic Epidemiology, International Agency for Research on Cancer, Lyon.

0002-9297/97/6101-0018$02.00

viduals with breast tumors that were thought to be benign. The family was published in a classic paper by Gardner and Stephens (1950), one of the earliest reports of a well-documented high-risk breast cancer family. It has since been updated a number of times, notably by Bishop and Gardner (1980) and Bishop et al. (1988). Since the original publication by Gardner and Stephens (1950), a total of 30 cases of breast cancer have occurred either in the individuals in the original pedigree or in their descendents, including 3 cases of breast cancer in men (see fig. 1). Affected individuals in this family have now been shown to carry a germ-line 2-bp deletion at nucleotide 277 (codon 17) in the BRCA2 gene (Tavtigian et al. 1996).

Kindred CRC186 was originally ascertained by one of us (P.A.D.) at Trinity Hospital, Dublin, on the basis of five affected sisters all diagnosed with breast cancer at age <50 years. The family has been studied extensively in Ireland by three of us (P.A.D., W.O., and D.A). Aside from the initial sibship, a total of 11 female breast cancers (all but 1 diagnosed at age <50 years) and 1 male breast cancer have been identified by sequential extension of the pedigree (see fig. 2). Affected individuals in this family have been shown to carry a nonsense mutation at nucleotide 9179 (codon 2984).

For this analysis, both families were extended to include all second-degree relatives of any female breast cancer case diagnosed at age <60 years or of any male breast cancer case diagnosed at any age, in a line of descent consistent with autosomal dominant inheritance. This process was repeated sequentially until no further relatives could be added. For kindred UTAH107, which was ascertained on the basis of a cluster of cases and then was followed prospectively, this process effectively included a subset of descendents of a founder couple (indicated by plus signs in fig. 1) born in 1819 and 1823. For kindred CRC186, which was ascertained on the basis of a more recent sibship, the extension led to the inclusion of both previous and subsequent generations and lateral branches. Diagnoses were confirmed, where possible, by histology reports, death certificates, or cancer-registration records.

Marker Typings

Individuals were genotyped for nine microsatellite markers—namely, D13S289, D13S290, D13S260, D13S171, D13S267, D13S220, D13S219, D13S218, and D13S263—spanning the BRCA2 region on chromosome 13q. Details of the genotyping methods used have been described by Cannon-Albright et al. (1992) and Stratton et al. (1994). Linkage results for the two families have been reported in full by Wooster et al. (1994). Multipoint linkage analysis based on the markers D13S260 and D13S267 and on the disease gave LOD scores of 3.70 for kindred CRC186 and 3.48 for kindred UTAH107.

Statistical Analysis

The age-specific risks of breast cancer in BRCA2 carriers were estimated by maximizing the following conditional log likelihood: $l = \log[L(\text{disease + marker phenotypes} \mid \text{ascertained pedigree})] = l(D,M \mid Asc;\beta)$. Here M represents the marker phenotypes, Asc the disease phenotypes involved in the initial ascertainment of the pedigree, D the disease phenotypes not part of the initial ascertainment, and β the penetrance parameters. Conditioning on the portion of the pedigree used in the initial ascertainment, which follows the standard procedure in segregation analysis, is necessary to correct for the fact that the family was initially ascertained on the basis of a cluster of several affected individuals. Omitting this correction would result in an overestimate of the penetrance.

It is not immediately apparent that maximizing l is valid, since the selection of individuals for marker typing must, to some extent, be dependent on their disease status (e.g., it might be more difficult to sample an affected individual because he or she has died). However, l can also be written as

$$1(\beta) = \log[L(D,Asc \mid Asc;\beta)] + \log[L(M,D,Asc \mid Asc;\beta)] = l_1(\beta) + l_2(\beta),$$

where $l_1(\beta)$ is the log likelihood of all the observed disease phenotypes, given the ascertainment but with the marker data being ignored, and $l_2(\beta)$ is the log likelihood of the marker data, given the disease phenotypes, which is equivalent to the LOD score (plus a constant that is independent of the parameter values).

That $l(\beta)$ will give consistent estimates of β follows directly from the fact that both $l_1(\beta)$ and $l_2(\beta)$ individually give consistent estimates of β. The fact that $l_1(\beta)$ gives consistent estimates of β follows from the fact that the pedigrees have been extended according to a strict sequential sampling rule, in this case including all relatives, up to the second degree, of affected individuals (Thompson 1986). The only uncertainty here is whether the correct portion of each pedigree has been designated as being involved in the ascertainment, so there is some approximation in this ascertainment procedure. However, since a large majority of each pedigree is clearly not part of the ascertainment, any such error will make little difference to the results.

The fact that maximizing $l_2(\beta)$ (which is equivalent to maximizing the LOD score) gives consistent estimates of β has been shown by Clerget-Darpoux and Bonaïti-Pellié (1992), among others. The advantage of maximizing $l(\beta)$ rather than $l_1(\beta)$ or $l_2(\beta)$ is that it incorporates all

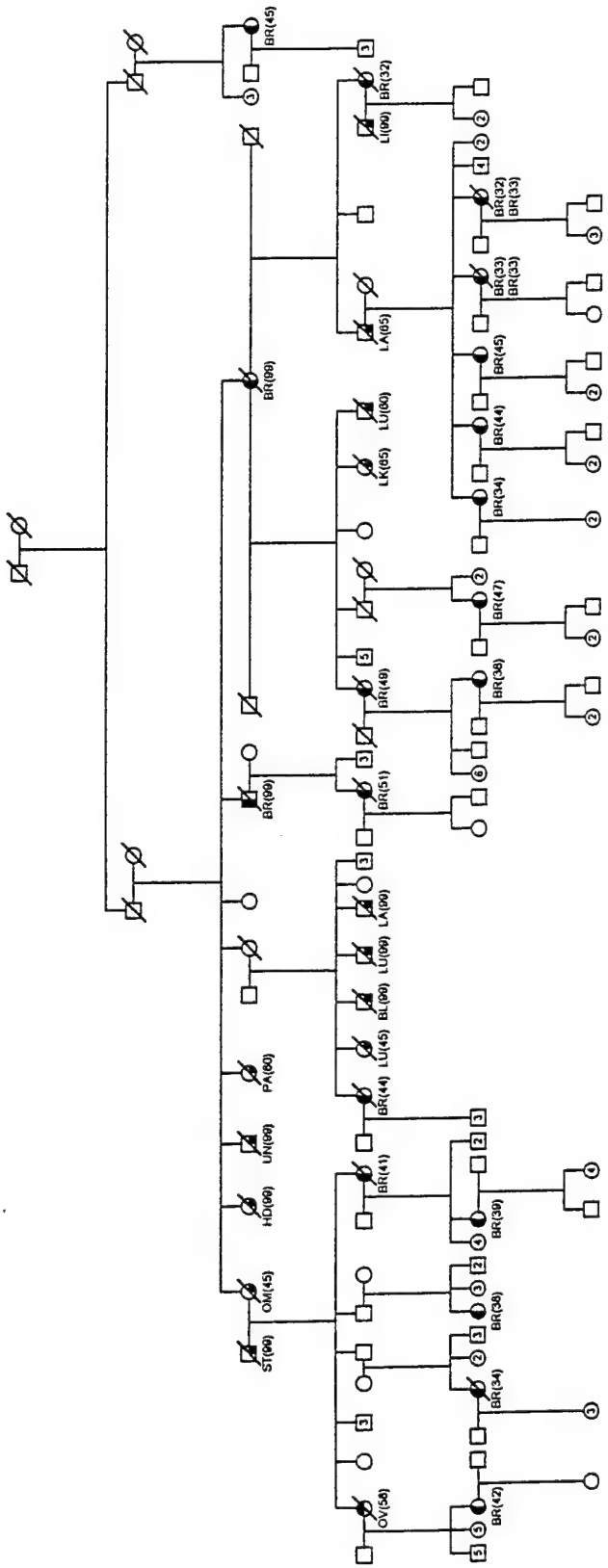

Figure 2 Pedigree of kindred CRC186. BL = bladder; HD = Hodgkin disease; LA = larynx; LI = liver; OM = ocular melanoma; and PA = pancreas; all other symbols and abbreviations are as in figure 1. Carrier status has been omitted to preserve confidentiality.

Table 2

Number of Cancers, Other than Breast Cancer, in UTAH107 and CRC186; Expected Number of Cancers at National Rates; and Estimated Relative Risks in BRCA2 Carriers

Cancer Site	No. of Cancers Observed/ No. of Cancers Expected			Estimated Relative Risk
	Carriers	Noncarriers	Unknown	
Stomach	0/.16	0/.11	0/.24	...
Colon/rectum	1/.72	0/1.58	0/2.85	.70
Larynx	1/.09	0/.16	1/.27	7.67*
Lung	0/.94	0/1.79	5/3.12	.81
Melanoma	0/.17	2/2.32	0/.43	...
Uterus, exclusive of cervix	1/.13	3/.48	1/.62	2.37
Ovary	3/.11	2/.27	0/.37	17.69**
Prostate	4/.63	1/1.12	1/2.21	2.89*
Leukemia	0/.16	0/.28	2/.51	.81
Other	0/1.69	1/3.82	4[a]/2.56	.28
Overall	10/5.10	9/9.93	14/17.12	1.27

[a] One each of cancer of the bladder, kidney, and pancreas and Hodgkin disease.
* $P < .01$.
** $P < .001$.

considered separately are very similar. Note that the risk estimates at age >60 years are very uncertain, owing to the small number of gene carriers followed beyond that age. The data are in fact also consistent with a model in which the incidence rates in BRCA2 carriers are equal to general population rates at age >60 years (twice the log-likelihood difference = 0.94). The estimated cumulative risk for male breast cancer by age 70 years is 6.3% (95%CI 1.4%–25.6%), or a relative risk ~150-fold the general population rates. There is some suggestion that the relative risk of female breast cancer in BRCA2 carriers, compared with that in the general population, declines with age, although the decrease in relative risks with age is not significant ($\chi^2_1 = 0.75$ for difference, in relative risk, between age <50 years and age >50 years). Under the model in table 2, the relative risks in age groups 30–39 years, 40–49 years, 50–59 years, 60–69 years, and 70–79 years are 36.3, 67.9, 21.2, 18.7, and 1.0, respectively.

Risks of Cancers Other than Breast Cancer

Table 2 shows observed and expected numbers of cancers other than breast cancer that have occurred since 1950 in individuals who were inferred to be BRCA2 carriers, noncarriers, or first-degree relatives of breast cancer cases whose carrier status could not be inferred. Significant excesses were observed for ovarian cancer (relative risk 17.69, $P < .001$), laryngeal cancer (relative risk 7.67, $P < .01$), and prostate cancer (relative risk 2.89, $P < .01$). Both of the laryngeal cancer cases in possible carriers occurred in kindred CRC186, whereas all the prostate cancers occurred in kindred UTAH107.

In addition to the cancers in table 2, several cancers were diagnosed in obligate or potential gene carriers before 1950. The most intriguing observation is that both families contain one obligate carrier who developed an ocular cancer. In kindred CRC186 this malignancy has been confirmed as a melanoma of the choroid of the eye, diagnosed at age 45 years. Ocular melanoma (at age 56 years) is also a plausible diagnosis for individual 109 in kindred UTAH107, although this occurred in 1905 and cannot be confirmed. Since both of these cancers occurred before 1950, when cancer-incidence data must have been seriously incomplete, it is not possible to state precisely what the corresponding expected numbers should be. However, even if complete cancer-incidence data before 1950 were available, the total expected number of cancers of the eye in either obligate carriers or first-degree relatives would still be only ~.06 ($P = .002$).

Discussion

These analyses confirm that germ-line BRCA2 mutations, at least in these two families, confer a high lifetime risk of breast cancer in women. The estimated cumulative risk of breast cancer is very similar, at all ages, to that estimated for BRCA1 carriers; using data collected by the Breast Cancer Linkage Consortium, Easton et al. (1995) estimated the risk of breast cancer in BRCA1-mutation carriers to be 51% by age 50 years, which compares with the 60% estimated for BRCA2 in this study. The corresponding estimates by age 60 years are 54% for BRCA1-mutation carriers, compared with 71% for BRCA2.

observed versus 9.93 expected) indicates that comparison with population rates is justified. It should also be noted that one of the ovarian cancer cases occurring in noncarriers (in kindred UTAH107) was nonepithelial in origin. Excess risks of ovarian cancer have also been noted, anecdotally, in other BRCA2 families (Wooster et al. 1995; Neuhausen et al. 1996; Thorlacius et al. 1996). The observed relative risk would correspond to absolute risk of ~10% by age 60 years, compared with the cumulative risk of 30%, estimated by Easton et al. (1995), under the assumption that all BRCA1 mutations confer the same risk. Our estimate for BRCA2 is, however, quite similar to the estimated risk associated with "low ovarian cancer risk" BRCA1 mutations under Easton et al.'s allelic-heterogeneity model (11% by age 60). However, it should be noted that another BRCA2-linked kindred, UTAH2044, reported by Wooster et al. (1994), contains five ovarian cancers, six female breast cancers diagnosed at age <50 years, and one male breast cancer case; one of the ovarian cancers was diagnosed, in a known carrier, since that publication. This suggests that, as for BRCA1, a subset of BRCA2 mutations will confer a more substantial ovarian cancer risk. In addition to the excess of ovarian cancer and the two reported eye cancers, which seem likely to be caused by BRCA2, our results also suggest, tentatively, an excess risk of two other cancers in BRCA2 carriers—namely, laryngeal cancer and prostate cancer. It is important to emphasize that these excesses, although formally significant, are based on very limited data (with only one case of laryngeal cancer and four cases of prostate cancer in proved carriers); the excess of laryngeal cancer is particularly uncertain, since there is no excess risk of cancer in carriers once ovarian cancer and prostate cancer have been accounted for (3 observed vs. 4.36 expected). More-definitive evidence on the risks of these cancers will be obtained only from collaborative analyses of much larger series of linked families, as is currently being undertaken by the Breast Cancer Linkage Consortium. However, it is interesting to note that significant excesses of both laryngeal cancer and prostate cancer have been observed in systematic studies of the relatives of early-onset breast cancer patients (Tulinius et al. 1992; Peto et al. 1995), and that an excess of prostate cancer has been specifically observed in BRCA2 families from Iceland (Thorlacius et al. 1996). It is also important to emphasize that the data do not exclude moderate relative risks of several other cancers (such as have been reported for colon and prostate cancer in BRCA1 carriers [Ford et al. 1995]), although the results do indicate that the overall excess of cancers other than breast and ovarian cancer is probably not large. Again, more-reliable estimates of risks should be obtained once larger numbers of families have been analyzed.

Acknowledgments

The analyses presented in this paper were conducted while D.F.E. was on sabattical at the University of Utah. We would particularly like to acknowledge the work of the late Dr. Eldon Gardner and his colleagues, in extending and updating kindred UTAH107 over 4 decades. This work was supported by the Cancer Research Campaign.

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GENETIC TESTING
Volume 1, Number 2, 1997
Mary Ann Liebert, Inc.

Mutation Testing of Early-Onset Breast Cancer Genes *BRCA1* and *BRCA2*

SUSAN L. NEUHAUSEN[1] and ELAINE A. OSTRANDER[2]

ABSTRACT

***BRCA1* and *BRCA2*, genes predisposing to early-onset breast cancer, have been isolated and are characterized for mutation spectrum, risks of cancer, and function. The different methodologies to screen for mutations in *BRCA1* and *BRCA2* are briefly discussed including DNA-based methodologies and potential new assays. The numbers and types of mutations identified to date are described, including the problems of ascribing risk to missense mutations. Recurring, possibly founding mutations have been identified in several populations including Ashkenazi Jews, Icelanders, Swedes, and African Americans. From population-based studies, estimates are that 6%–10% of breast cancers are due to mutations in *BRCA1* and *BRCA2*. Knowledge of mutation status raises additional questions including the interpretation of negative tests and the risks of breast and other cancers associated with positive test results.**

BREAST CANCER IS A WIDESPREAD DISEASE. In the United States, current estimates are that 1 in 8 women will be affected with the disease at some point during their lives (American Cancer Society, 1996). Although a number of environmental variables appear to affect risk for breast cancer, a positive family history of the disease has been identified as one of the most significant risk factors. Women who have a first-degree family history of the disease have a 2- to 10-fold increased risk over women without a strong family history (Brinton *et al.*, 1982; Ottman *et al.*, 1986; Sattin *et al.*, 1985; Cannon-Albright *et al.*, 1991). Age of onset is a second strong indicator of familial breast cancer risk (Claus *et al.*, 1990).

Genetic predisposition may explain a significant proportion of early-onset breast cancer. Estimates are that approximately 7% of breast cancer cases and 10% of ovarian cancer cases in the general population are caused by mutations in autosomal dominant, highly penetrant, breast cancer susceptibility genes (Claus *et al.*, 1996). Two of these genes, *BRCA1* and *BRCA2*, have been isolated (Miki *et al.*, 1994; Wooster *et al.*, 1995) and over 200 mutations have been identified (Breast Cancer Information Core). The availability of sequence information from both *BRCA1* and *BRCA2* has allowed for the development of diagnostic protocols for screening and identification of predisposing germline mutations. As a result, among women with significant family histories of breast cancer, there is considerable interest regarding the utility and availability of genetic tests. Genetic tests ideally should provide an accurate diagnosis of predisposition accompanied by detailed information on the risk associated with each mutation and how this risk may be modified. In this review, we discuss what is known about *BRCA1* and *BRCA2* in terms of mutation detection, types of mutations, risks for cancer, and considerations for genetic testing.

MUTATION SCREENING METHODOLOGIES

Several key issues that need consideration in the development of *BRCA1* and *BRCA2* testing protocols include: (1) the sensitivity of the screening technique; (2) the significance of missense mutations to cancer predisposition; (3) the frequency and significance of mutations in noncoding regions of the gene; (4) the ability to assign specific risk to each mutation; (5) the interpretation of a "negative" test in a women whose family history puts her at risk. Several types of testing strategies are currently in use or under development.

[1]Division of Genetic Epidemiology, Department of Medical Informatics, University of Utah School of Medicine, 410 Chipeta Way, Room 105, Salt Lake City, UT 84108.
[2]Clinical Research Division M318, Fred Hutchinson Cancer Research Center, Seattle, WA 98104.

UTILITY OF DNA BASED APPROACHES

For individuals with a strong family history of breast cancer and multiple affected living relatives, linkage analysis, and/or analysis of haplotype sharing at *BRCA1* and *BRCA2* can be used as an initial step prior to mutation screening. DNA is obtained from peripheral blood samples of all living affected women as well as several other relevant family members. Analysis of genotypes is performed using polymorphic microsatellite repeat markers that define the *BRCA1* region on 17q and the *BRCA2* region on 13q. The presence of shared haplotypes among affected women can be helpful to the trained counselor in assessing a woman's risk. However, this method will not identify the mutation, just the likelihood that cancer in a family is due to a predisposing mutation in *BRCA1* or *BRCA2*.

One difficulty in selecting a specific technique for mutation testing is the large size and relative complexity of the *BRCA1* and *BRCA2* genes. A variety of technologies are available to detect germline mutations. However, only a small subset of these are suitable for genetic testing. When screening large populations to determine the population frequency of mutations, detection methods such as single-stranded conformation polymorphism analysis (SSCP) (Orita *et al.*, 1989), SSCP combined with heteroduplex analysis (SSCA/HA) (Gayther *et al.*, 1996), conformation sensitive gel electrophoresis (CSGE) (Ganguly *et al.*, 1993), and constant denaturing gel electrophoresis (CDGE) (Boerresen *et al.*, 1991) are suitable. Such techniques rely on detecting a variant band in a gel-based system. Direct sequencing of the anomalously migrating band is used to identify the precise DNA mutation. Because single-base changes often cause only small perturbations in these assays, methods like SSCP and CDGE are imperfect, detecting about 80% of mutations in controlled studies (Jordanova *et al.*, 1997).

Direct DNA sequencing of the coding region and intron/exon boundaries of the gene is the most reliable approach with the highest specificity and sensitivity. However, it is time consuming, expensive, and still imperfect because sequencing enzymes occasionally produce artifacts. Some investigators have used this technique to determine *BRCA1* and *BRCA2* mutation frequency in young women from the general population (FitzGerald *et al.*, 1996) and it is the method currently used by the commercial testing company Myriad Genetics Laboratories.

The protein truncation test (PTT) can be performed if it is possible to obtain RNA from patients (Roest *et al.*, 1993). This approach, which has been widely used to detect truncating mutations in the *BRCA1* and *BRCA2* genes, is considered to be quick and efficient (Hogervorst *et al.*, 1995; Plummer *et al.*, 1995). One disadvantage is that it does not detect missense changes, so that single-base substitutions that are known to cause disease, such as the *BRCA1* Cys61Gly mutation would not be detected. For that reason, PTT is probably not suited for use in a genetic testing setting unless it is utilized with another method that at least detects those missense mutations that are known to be disease-associated.

One shortcoming of all current DNA-based mutation detection methodologies is that they are not sensitive to large deletions or to splice mutations that remove entire exons. Splice mutations that are outside of the amplicons examined will be missed even by direct sequencing. Second, without RNA, it is difficult to predict whether the splice mutation will cause the removal of an exon or insertion of an intron. Southern blotting can be utilized to detect large deletions, insertions, and other rearrangements, but it is time consuming and requires a large amount of DNA. Large deletions may also be detected through quantitative PCR.

SENSITIVITY OF CURRENT APPROACHES

The most difficult case to evaluate is that of a woman perceived to be at risk based on anecdotal histories of other family members when affected family members are unavailable for testing. Whereas complete testing utilizing a combination of techniques that involve screening for point mutations, frameshift mutations, splice mutations, and large deletions and other rearrangements can be used, there may still be alterations in levels of mRNA resulting from changes in regulatory regions that will not be identified by the aforementioned techniques. Currently, there is insufficient knowledge about the functions of *BRCA1* and *BRCA2* to determine the frequencies of such mutations and their significance in disease predisposition. Hence, it is nearly impossible to give negative *BRCA1* and *BRCA2* test results to women and be certain that no alterations have been missed.

It is easier to evaluate a family member perceived to be at risk because a *BRCA1* or *BRCA2* mutation is already known to be segregating in the family. Once such a mutation has been identified, other family members can be tested for the presence or absence of that single mutation. Similarly, if a women is believed to be at risk because of ethnic status, she can be tested for the presence or absence of those mutations found frequently in that particular ethnic group. Typically, the region containing the mutation is amplified from DNA isolated from the patient's peripheral blood sample and the resulting product is assayed by allele-specific oligonucleotide (ASO) assay, gel-based assays, and/or sequencing. All of these approaches have been used in large-scale screening of Ashkenazi Jewish women who are at particularly high risk for a 185delAG mutation in *BRCA1* and a 6174delT mutation in *BRCA2* (Struewing *et al.*, 1995; Abeliovich *et al.*, 1997). A problem with testing only for the specific mutation is that a family member may carry a different mutation. In that case, she would be counseled that her test is negative and that she is at no greater risk then the population, when she actually is at increased risk. Such cases are rare. In one example, a mother had a frameshift mutation and her daughter had a different frameshift mutation inherited from her father (Stoppa-Lyonnet *et al.*, 1996).

FUNCTIONAL ASSAYS AND NEW METHODOLOGIES

Ishioka *et al.* (1997) have been able to detect heterozygous-truncating mutations in both the *BRCA1* and *APC* genes. In their system, a PCR amplified coding sequence is inserted via homologous recombination into the URA3 gene in yeast and growth is assayed. Those transformants that harbor an inserted stop codon will grow poorly on media lacking uracil. This assay, while providing accurate and reliable information regard-

ing frameshift and nonsense mutations, is not useful for detecting missense mutations.

A promising test with sensitivity to missense changes was described by Humphrey *et al.* (1997) in which growth inhibition of yeast colonies is examined. Growth of yeast is inhibited by expression of wild-type *BRCA1* and is not inhibited by expression of *BRCA1* containing frameshift, nonsense, or disease-associated missense mutations in the C terminus of the protein. This assay system offers the advantage that it has the potential to detect missense mutations, although more characterization of the test needs to occur before it is used in clinical screening.

A third new technology, whose feasibility is being tested for mutation detection, uses oligonucleotide arrays in a DNA chip-based assay (Hacia *et al.*, 1996). Chip technologies allow for rapid screening for a large number of possible mutations. In one application, a high-density array of approximately 96,600 20-bp oligonucleotides was used to screen for mutations in the 3.5 kb exon 11 of the *BRCA1* gene. In this analysis, 14 of 15 samples with known mutations were accurately detected, no false positives were seen in 20 control samples, and 8 of 8 single-based changes (missense mutations) were accurately identified. The only undetected mutation was an expansion of a poly (dA).(dT) tract from 7 to 8 nucleotides in length. The most suitable methodology for genetic testing will be dependent on the needs of the patient, the number and cancer status of participating family members, and the information already available on mutation status in her family.

MUTATIONS REPORTED TO DATE

Mutations in the *BRCA1* and *BRCA2* genes are found uniformly throughout both genes and consist of insertion and deletion frameshifts, base substitutions (both nonsense and missense), splice variants, and regulatory mutations. Changes in the DNA sequence that are readily classified as disease-causing are divided largely into frameshift and nonsense mutations. Such changes, which account for the majority of *BRCA1* and *BRCA2* mutations observed thus far, cause truncation of the translated protein, and with the exception of the *BRCA2* Lys3326ter (Mazoyer *et al.*, 1996), have all been considered to be predisposing mutations. An international database, the Breast Cancer Information Core or BIC (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/) was created for scientists to record the mutations they have detected and technologies they utilize (Couch *et al.*, 1996; BIC).

One difficulty in determining genetic risk has been in assessing the contribution of rare missense changes that result in alteration of only a single amino acid. Thus far, only a few missense mutations are known to be disease associated. For example, the change of a cysteine to a glycine at amino acid 61 in *BRCA1* affects the RING finger motif in the protein and segregates with affected women in many high-risk families (Friedman *et al.*, 1994). The status of the remaining missense mutations that have been labeled in a variety of ways including "rare variants" or "missense changes of unknown consequence" will remain uncertain until functional assays for *BRCA1* and *BRCA2* are available. In the absence of a functional assay, characteristics which suggest that the missense mutation is predisposing are: (1) the mutation is not observed in a control group; (2) there is cosegregation of the variant and disease in a family with significant disease; (3) the missense change results in replacement of a dissimilar amino acid to the wild-type protein; (4) there is conservation of the wild-type amino acid between the human, mouse, and dog *BRCA1* or *BRCA2* proteins; (5) the mutation occurs in a putative functional domain. An additional complexity that may emerge as missense mutations are characterized is that their penetrance (i.e., the frequency with which they produce disease) may be less than that observed in truncating mutations.

Szabo and King (1995) suggested that 15%–20% of mutations affect expression, splicing, or stability of the transcript. The commonly used methods of mutation detection may be insensitive to these mutations. An estimate of the number of *BRCA1* mutations that are deletions may be calculated from the data of the Breast Cancer Linkage Consortium. *BRCA1* mutations were identified in 21 of 30 (70%) families linked to *BRCA1* (D. Easton, personal communication). There was loss of transcription from the cancer-associated allele in 2 of the remaining 9 families. In 1 of these families, a 14-kb deletion (caused by inter-Alu recombination) which removed the promoter region has been identified (Swensen *et al.*, 1997). In a third family, an Alu-mediated deletion of exon 17 was identified (Puget *et al.*, 1997). The other 6 families were not characterized, so a percentage of them also may have large deletions. These results suggest that 10%–30% of germline mutations in *BRCA1* may be large deletions. The potential presence of such deletions clearly needs to be considered when screening individuals for *BRCA1* and *BRCA2* germline mutations so that a false-negative test does not result.

FOUNDER EFFECTS

Several recurring *BRCA1* and *BRCA2* mutations have been identified. These appear to derive from common founders in specific populations. The first *BRCA1* and *BRCA2* founder mutations to be described were the *BRCA1* 185delAG and 5382insC and the *BRCA2* 6174delT mutations that were all identified in Ashkenazi Jews (Struewing *et al.*, 1995; Tonin *et al.*, 1995; Neuhausen *et al.*, 1996a). The two *BRCA1* mutations occur in approximately 1%–1.5% and the *BRCA2* mutation in 1%–1.5% of Ashkenazi Jews (Table 1). This is in stark contrast to estimates of 1/800–1/1500 mutation carrier frequency for *BRCA1* that has been reported for the general Caucasian population (Claus *et al.*, 1991; Ford *et al.*, 1995). These three mutations have been examined in multiple cohorts of Jewish women with and without family histories of breast and/or ovarian cancer (Table 1). Interestingly, the *BRCA2* 6174delT mutation occurs with the same or a slightly higher frequency in the Ashkenazi Jewish population, yet it accounts for fewer of the cases of breast cancer (Table 1), suggesting lower penetrance. However, in a population-based study of Ashkenazi Jews, Struewing *et al.* (1997) saw no significant differences in penetrance for these mutations. In Ashkenazi Jewish women, these three mutations appear to account for approximately 30% of breast cancer diagnosed at any age (Tonin *et al.*, 1996; Abeliovich *et al.*, 1997) and 39% of ovarian cancer diagnosed in women less than 50 years of age (Struewing *et al.*, 1995; Muto *et al.*, 1996), suggesting that

TABLE 1. FREQUENCY OF *BRCA1* AND *BRCA2* MUTATIONS IN ASHKENAZI JEWS

Source	*N*	*185delAG*	*5382insC*	*6174delT*	*References*
Pop'n-based	varied	0.8–1.1%	0.13–0.3%	0.9–1.5%	Struewing, 1995; Roa, 1996; Oddux, 1996
BC < age 42	80	20.0%	4.0%	8..0%	Offit, 1996; Neuhausen, 1996
BC 42–50 yrs	27	30.0%	4.0%	7.0%	Offit, 1996; Neuhausen, 1996
BC only families	138	20.0%	5.0%	4.0%	Tonin, 1996
B/O families	82	52.0%	16.0%	5.0%	Tonin, 1996

screening for these specific mutations may be useful for genetic counseling in this population.

Other recurring mutations have been observed in other populations (Table 2). Only the Icelandic *BRCA2* 995del5 mutation has been well-characterized (Table 3). This mutation occurs with a frequency of 0.6% in the Icelandic population and explains as much as 40% of male breast cancer in that population (Table 3). The majority of the breast cancer studies have examined women of Northern European ancestry. African-American women, who have a higher incidence of early onset breast cancer (Kosary *et al.*, 1995), have not been well studied to date. However, data for *BRCA1* are suggestive that African Americans have different mutations than Caucasians and that there also may be founder effects in this population. Recently, three novel *BRCA1* mutations were identified in 5 of 9 (56%) African-American families screened for mutations (Gao *et al.*, 1997). Haplotypes were generated in the families with the recurrent mutations and the women who carried the same mutation shared the same haplotype in the *BRCA1* region suggestive of a founder effect.

FREQUENCY OF *BRCA1* AND *BRCA2* MUTATIONS

In a recent analysis of the Breast Cancer Linkage Consortium (BCLC), it was estimated that, of 237 families with at least 4 cases of breast cancer, 52% are due to *BRCA1*, 32% to *BRCA2*, and 16% to other genes. In those families with 5 or fewer cases of breast cancer and no ovarian or male breast cancers, less than 40% of the families can be explained by linkage to *BRCA1* and *BRCA2* (Easton, personal communication). Studies of site-specific breast cancer families for which few *BRCA1* and *BRCA2* mutations have been identified confirm this result (Serova *et al.*, 1997; Schubert *et al.*, 1997). Thus, searches are underway in non-*BRCA1*/*BRCA2* families to identify new breast cancer genes.

Studies of selected cohorts of families are useful for modeling risk of cancer, but they do not provide information about the population frequency of *BRCA1* or *BRCA2* mutations nor do they address the issue of mutant allele frequency in women who are not from high-risk families. Thus, theoretical analyses have been very useful. Whittemore *et al.* (1997) and Ford *et al.* (1995) pooled data from population-based case-control studies to estimate the proportions of breast and ovarian cancers due to *BRCA1* and *BRCA2*. Whittemore *et al.* (1997) estimated that 4.2% of breast cancer and 5.3% of ovarian cancer diagnosed by age 70 and 11% of breast cancer diagnosed before age 40 were due to inherited mutations. The results of Ford *et al.* (1995) were more conservative, estimating that 5.3% of breast cancers diagnosed before age 40, 2.2% diagnosed between ages 40 and 49, and 1.1% diagnosed between ages 50–70 were likely due to mutations in *BRCA1*, *BRCA2*, and other similar highly penetrant genes. The differences in the estimates likely reflect differences in the data sets and the assumptions of the models.

These theoretical estimates are similar to results obtained from mutation screening of a population-based sample of women with breast cancer. In two different studies that focused exclusively on age of onset as a predictor of *BRCA1* status, 8% and 10% of women younger than ages 35 and 30 years, respectively, were found to carry germline mutations in the *BRCA1* gene (FitzGerald *et al.*, 1996; Langston *et al.*, 1996). In each case, women were selected based only on age of onset of breast cancer and not for family history status. Interestingly, several of the women who were shown to be *BRCA1* carriers in these studies, lacked a significant family history of breast cancer. These data underscore the fact that it will be nearly impossible to identify all women at risk based solely upon family history criteria. The situation appears similar with *BRCA2* although data from both population-based and family-based

TABLE 2. RECURRENT MUTATIONS IN OTHER POPULATIONS

Population	*Mutation*	*Citation*
Italians from Tuscany	*BRCA1*–1499insA	Caligo *et al.*, 1996
Icelandics	*BRCA2*–999del5	Thorlacius *et al.*, 1996
Japanese	*BRCA1*–L63X	Inoue *et al.*, 1995
Southern Swedes	*BRCA1*–Q563X	Johannsson *et al.*, 1996
	BRCA1–3166ins5	Johannsson *et al.*, 1996
	BRCA1–1201del11	Johannsson *et al.*, 1996
African Americans	*BRCA1*–Met1775Arg	Miki *et al.*, 1994
	BRCA1–1832del5	Gao *et al.*, 1997
	BRCA1–5296del4	Gao *et al.*, 1997

TABLE 3. PREVALENCE OF THE ICELANDIC *BRCA2* FOUNDER MUTATION 995DEL5

Group	*N*	*995del5+*	*Shared haplotype*	*Comments*	*References*
Pop'n-based	520	3 (0.6%)	—		Thorlacius *et al.*, 1997
Families	21	16 (76%)	Yes	9/16 had MBC	Thorlacius *et al.*, 1996
MBC[a] cases	30	12 (40%)	Yes	In the 9 families	Thorlacius *et al.*, 1996
FBC[b] cases	632	49 (8%)	—		Thorlacius *et al.*, 1997
Prostate cancer[c]	77	10 (13%)	—	Sig. worse survival	Sigurdsson *et al.*, 1997

[a]MBC, male breast cancer.
[b]FBC, female breast cancer.
[c]Other cancers: Pancreas, ovary, colon, stomach, thyroid, cervix, endometrium.

studies suggest that mutations in *BRCA1* are 2–3 times more common than those in *BRCA2* (Krainer *et al.*, 1997; for review see Szabo and King, 1997). Recently, modeling has been performed to assess which women may want to consider being tested for *BRCA1* mutations (Berry *et al.*, 1997; Couch *et al.*, 1997). Some factors considered are family history of breast and ovarian cancers, population mutation frequencies, and ethnic origin. These models are being refined to include *BRCA2* in the estimates.

CANCER RISKS

For both *BRCA1* and *BRCA2* mutation carriers, the penetrance for breast and ovarian cancer is incomplete and not all mutation carriers will be affected with cancer. Based on estimates derived largely from Caucasian families of Northern European descent with a minimum of four cases of breast cancer, the cumulative risks for *BRCA1* and *BRCA2* mutation carriers is greater than 80% by age 70 for breast cancer (Easton *et al.*, 1995; Narod *et al.*, 1995; Easton, personal communication). In these same families, the overall risk of ovarian cancer ranges from 42%–63% (Easton *et al.*, 1993; Narod *et al.*, 1995; Easton *et al.*, 1995). However, there is significant heterogeneity for ovarian cancer risks so that in 89% of those families the ovarian cancer risk by age 70 is 26% and in the remaining 11% of families, the risk is 85% (Easton *et al.*, 1995). This suggests that different mutations may confer quantitatively different risks of ovarian cancer.

At this time, there are little data to suggest mutation-specific effects. In one study, a comparison of the relative proportions of cases of breast and ovarian cancer among six recurring *BRCA1* mutations was suggestive of such an effect ($p = 0.069$), where 57% (26/88) of women with the 1294del40 variant had ovarian cancer compared to 14% (6/42) of women with the intron5-splice variant (Neuhausen *et al.*, 1996b). Genotype–phenotype correlations have been suggested for both *BRCA1* and *BRCA2*, based on clustering of mutations with excesses of ovarian cancers in families. For *BRCA1*, there is a correlation between decreased ovarian cancer risk and mutations occurring after codon 1443 (nucleotide 4447) (Gayther *et al.*, 1995). These data fit well with the observation by Thompson *et al.* (1995) that 3′ mutations in *BRCA1* did not inhibit ovarian cancer cell growth, whereas 5′ mutations did. One exception to this, however, is the high incidence of ovarian cancer in carriers of the 5382insC mutation (Tonin *et al.*, 1996; Gayther *et al.*, 1997a), which may be due to this mutation or to an uncharacterized founder effect from another gene.

For *BRCA2*, ovarian cancer risk is estimated to be 27% by age 70 (Easton, personal communication), which is not as high as for *BRCA1*. Gayther *et al.* (1997b) proposed a *BRCA2* ovarian cancer cluster region (OCCR) in the middle of exon 11 between nucleotides 3035 and 6629. Support for the existence of an OCCR region may be due largely to the elevated risk of ovarian cancer with the Ashkenazi Jewish 6174delT mutation. In ovarian cancer cases, this mutation has been found with a similar frequency to the *BRCA1* 185delAG mutation (Abeliovich *et al.*, 1997; Levy-Lahad *et al.*, 1997).

BRCA1 and *BRCA2* carriers also appear to be at elevated risk for cancers other than breast and ovary. For *BRCA1*, estimates of a 4-fold increased risk for colon cancer and a 3-fold increased risk for prostate cancer have been reported (Ford *et al.*, 1994). However, in a study examining germline *BRCA1* mutations in men less than age 65 with prostate cancer, only 1 of 49 cases carried a predisposing mutation (Langston *et al.*, 1996). For *BRCA2* carriers, there appears to be an increased risk of pancreas, prostate, and male breast cancer, as well as ocular melanoma (Couch *et al.*, 1996; Thorlacius *et al.*, 1996; Easton *et al.*, 1997). In two studies, germline *BRCA2* mutations were identified in 3 of 41 (7.3%) and 2 of 41 (4.9%) of pancreatic cancer cases (Goggins *et al.*, 1996; Ozcelik *et al.*, 1997), suggesting that a significant proportion of pancreatic cancer may be attributed to this gene. Additional studies are needed to assess the risk for nonreproductive cancers in both *BRCA1* and *BRCA2* carriers, before it will be possible to provide patients with accurate risk data.

IMPLICATIONS OF GENETIC TEST RESULTS

Knowledge of mutation status is only one part of risk assessment. While it is possible to directly sequence both the *BRCA1* and *BRCA2* genes from women, a negative result can not be given with absolute certainty and may have several interpretations. First, the woman may carry no predisposing gene to breast cancer and is therefore at the same risk as the general population. Second, she may have a mutation in an as yet undiscovered gene predisposing to breast cancer, i.e., BRCA3, BRCAn. Third, she may carry a mutation in another gene, whose primary affect is not breast cancer but which causes an increased risk of breast cancer, i.e., *P53* (Malkin *et al.*, 1990; Boerresen *et al.*, 1991; 1992), ataxia telangiectasia (Savitsky

et al., 1995; Athma *et al.*, 1996; Bishop and Hopper, 1997; FitzGerald *et al.*, 1997), *PTEN/MMAC* (Liu *et al.*, 1997; Liaw *et al.*, 1997; Steck *et al.*, 1997). Alternatively, there may be weakly penetrant genes or autosomal recessive genes that contribute in some small part to breast cancer risk. Finally, a women may carry a *BRCA1* or *BRCA2* mutation which was undetected by the technology used for screening.

Once a woman tests positive for a *BRCA1* or *BRCA2* germline mutation, she will still be faced with making decisions regarding the unknown. The patient and her physician will need to consider what her personal risk is for breast and/or ovarian cancer, whether a prophylactic mastectomy and/or oophorectomy is appropriate; and if she does get breast cancer, whether lumpectomy or mastectomy is appropriate given her increased risk of a second breast cancer. Finally, the patient and her physician will need to decide if she should use estrogen replacement therapy when she is menopausal.

For both *BRCA1* and *BRCA2*, there is variable penetrance and differences in proportions of breast and ovarian cancers as well as other cancers, even among families with common mutations on the same haplotypic background (Simard *et al.*, 1994; Tonin *et al.*, 1996; Thorlacious *et al.*, 1996). Expressivity varies from early-onset bilateral breast cancer and ovarian cancer to late-onset breast cancer, and from no other cancers in the family to other cancers such as prostate, pancreas, etc. Therefore, it is not possible to assign mutation specific risks. This variation suggests that there are hormonal, environmental, and genetic factors which modulate age-specific risk and overall incidence of breast and ovarian cancers in mutation carriers. Identification of these factors in women with known *BRCA1* and *BRCA2* mutations may be important for individual risk assessment in order to counsel mutation carriers about potential means of reducing their cancer risks. In addition, there is still a great deal to be learned regarding the most appropriate treatment or prevention strategy for women who carry mutations.

For those involved in genetic counseling and risk assessment, uncertainty regarding the number and role of breast cancer genes clouds the determination of an individual's genetic predisposition to breast cancer. Perhaps it is best to keep in mind that since one in eight women in the industrialized world succumb to breast cancer at some point in their life, all women are at risk for developing breast cancer. For a small subset of women, better predictions about their likelihood of developing the disease early in life can be made. But all women, even those shown to be "not at risk" through genetic testing are indeed at risk by virtue of the fact they are women. The goal of genetic counseling will be to not only assess the risk of women from unique families, but to provide women throughout the community with the best and most up-to-date information available regarding our emerging knowledge of the genetics of breast cancer.

ACKNOWLEDGMENTS

S.L.N. is supported by U.S. Army Grants DAMD17–94–J–4260 and DAMD17–96–1–6266 and NIH Grant CA55914. E.A.O. is the recipient of National Cancer Institute Grant R01 CA63705 and American Cancer Society Junior Faculty Award JFRA–558.

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Address reprint requests to:
Dr. Susan L. Neuhausen
Division of Genetic Epidemiology
University of Utah
410 Chipeta Way, Room 105
Salt Lake City, UT 84108

Am. J. Hum. Genet. 62:676–689, 1998

Genetic Heterogeneity and Penetrance Analysis of the BRCA1 and BRCA2 Genes in Breast Cancer Families

D. Ford,[1] D. F. Easton,[3] M. Stratton,[2] S. Narod,[5] D. Goldgar,[6] P. Devilee,[7] D. T. Bishop,[8] B. Weber,[9] G. Lenoir,[6] J. Chang-Claude,[10] H. Sobol,[11] M. D. Teare,[3] J. Struewing,[12] A. Arason,[13] S. Scherneck,[15] J. Peto,[1] T. R. Rebbeck,[9] P. Tonin,[16] S. Neuhausen,[17] R. Barkardottir,[13] J. Eyfjord,[14] H. Lynch,[18] B. A. J. Ponder,[4] S. A. Gayther,[4] J. M. Birch,[19] A. Lindblom,[20] D. Stoppa-Lyonnet,[21] Y. Bignon,[22] A. Borg,[23] U. Hamann,[10] N. Haites,[25] R. J. Scott,[26] C. M. Maugard,[27] H. Vasen,[28] S. Seitz,[15] L. A. Cannon-Albright,[17] A. Schofield,[25] M. Zelada-Hedman,[20] and the Breast Cancer Linkage Consortium

Sections of [1]Epidemiology and [2]Molecular Carcinogenesis, Institute of Cancer Research, Sutton, United Kingdom; [3]CRC Genetic Epidemiology Unit, Institute of Public Health, and [4]CRC Human Cancer Genetics Research Group, University of Cambridge, Cambridge; [5]Center for Research in Women's Health, Toronto; [6]International Agency for Research on Cancer, Lyon; [7]Departments of Pathology and Human Genetics, University of Leiden, Leiden; [8]ICRF Genetic Epidemiology Laboratory, Leeds; [9]Departments of Medicine and Genetics, University of Pennsylvania, Philadelphia; [10]Deutsches Krebsforschungszentrum, Heidelberg; [11]Département d'Oncologie-Génétique/INSERM CRI9703, Institut Paoli Calmettes, Marseille; [12]Genetic Epidemiology Branch, National Cancer Insitute, Bethesda; [13]Laboratory of Cell Biology, University Hospital of Iceland, and [14]The Icelandic Cancer Society, Reykjavik; [15]Max-Delbrück-Centrum für Moleculäre Medizin, Berlin; [16]Departments of Medicine and Human Genetics, McGill University, Montreal; [17]Genetic Epidemiology Group, University of Utah, Salt Lake City; [18]Department of Preventative Medicine and Public Health, Creighton University School of Medicine, Omaha; [19]CRC Paediatric and Familial Cancer Research Group, Christie Hospital and Holt Radium Institute, Manchester; [20]Department of Clinical Genetics, Karolinska Institute, Stockholm; [21]Unité de Génétique Oncologique, Institut Curie, Paris; [22]Laboratoire d'Oncologie Moleculaire, Centre Jean Perrin, Clermont-Ferrand, France; [23]Department of Oncology, University Hospital, Lund, Sweden; [24]Medical Genetics, University of Aberdeen, Aberdeen; [25]Humangenetik, Kantonsspital, Basel; [26]Centre Réné Gauducheau, Nantes; and [27]Foundation for the Detection of Hereditary Tumors Leiden

Summary

The contribution of BRCA1 and BRCA2 to inherited breast cancer was assessed by linkage and mutation analysis in 237 families, each with at least four cases of breast cancer, collected by the Breast Cancer Linkage Consortium. Families were included without regard to the occurrence of ovarian or other cancers. Overall, disease was linked to BRCA1 in an estimated 52% of families, to BRCA2 in 32% of families, and to neither gene in 16% (95% confidence interval [CI] 6%–28%), suggesting other predisposition genes. The majority (81%) of the breast-ovarian cancer families were due to BRCA1, with most others (14%) due to BRCA2. Conversely, the majority of families with male and female breast cancer were due to BRCA2 (76%). The largest proportion (67%) of families due to other genes was found in families with four or five cases of female breast cancer only. These estimates were not substantially affected either by changing the assumed penetrance model for BRCA1 or by including or excluding BRCA1 mutation data. Among those families with disease due to BRCA1 that were tested by one of the standard screening methods, mutations were detected in the coding sequence or splice sites in an estimated 63% (95% CI 51%–77%). The estimated sensitivity was identical for direct sequencing and other techniques. The penetrance of BRCA2 was estimated by maximizing the LOD score in BRCA2-mutation families, over all possible penetrance functions. The estimated cumulative risk of breast cancer reached 28% (95% CI 9%–44%) by age 50 years and 84% (95% CI 43%–95%) by age 70 years. The corresponding ovarian cancer risks were 0.4% (95% CI 0%–1%) by age 50 years and 27% (95% CI 0%–47%) by age 70 years. The lifetime risk of breast cancer appears similar to the risk in BRCA1 carriers, but there was some suggestion of a lower risk in BRCA2 carriers <50 years of age.

Introduction

Mutations in a number of genes are now known to cause susceptibility to breast and/or ovarian cancer. In the context of high-risk families, the most important genes are BRCA1 (MIM 113705 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?113705]) and BRCA2 (MIM 600185 [http://www3.ncbi.nlm.nih.gov:80/htbin-

Received June 30, 1997; accepted for publication December 4, 1997; electronically published March 4, 1998.

Address for correspondence and reprints: Dr. Douglas Easton, CRC Genetic Epidemiology Unit, Strangeways Research Laboratories, Worts Causeway, Cambridge CB1 4RN, United Kingdom. E-mail: douglas.easton@srl.cam.ac.uk

0002-9297/98/6202-0023$02.00

post/Omim/dispmim?600185]). BRCA1 was localized to chromosome 17q by genetic linkage in 1990 (Hall et al. 1990) and subsequently was cloned in 1994 (Miki et al. 1994). Studies to date suggest that BRCA1 accounts for the majority of families containing multiple cases of breast and ovarian cancer (Easton et al. 1993; Narod et al. 1995*b*), for less than half the families containing breast cancer only (Easton et al. 1993), and for few families that include male breast cancer cases (Stratton et al. 1994). The risks conferred by BRCA1 have been estimated both from linkage data (Easton et al. 1995) and from the study of cancer incidence in carriers within families with disease believed to be linked to the gene (Ford et al. 1994). The cumulative risk of breast or ovarian cancer in female carriers is estimated to be >80% by age 70 years, although there is evidence that the ovarian:breast cancer risk ratio varies between families, with mutations toward the 5′ end of the gene conferring a relatively higher ovarian cancer risk (Easton et al. 1995; Gayther et al. 1995; Holt et al. 1996).

BRCA2 was localized to chromosome 13q in 1994 (Wooster et al. 1994) and was cloned in 1995 (Wooster et al. 1995; Tavtigian et al. 1996). In their study of 15 families with evidence against linkage to BRCA1, Wooster et al. (1994) estimated that disease was linked to BRCA2 in 74% of families, providing preliminary evidence that BRCA1 and BRCA2 together might account for most high-risk breast cancer families. There is particularly clear evidence that BRCA1 and BRCA2 together are likely to account for the majority of high-risk breast-ovarian cancer families. In a study of 145 breast-ovarian cancer families, there were 10 families with strong evidence against linkage to BRCA1 (multipoint LOD scores <-1.0); of these, 3 now have an identified BRCA1 mutation, and 7 have an identified BRCA2 mutation (Narod et al. 1995*a*, 1995*b*). A number of families with evidence of linkage to 13q and/or with identified BRCA2 mutations contain male cases. Easton et al. (1997) have estimated the risks of cancer in BRCA2 mutation carriers in the two largest families showing linkage in the original study. They found that the breast cancer risk in females was similar to that conferred by a BRCA1 mutation and estimated the risk in males to be 6% by age 70 years. The increased risk of ovarian cancer in mutation carriers appears to be lower in most families than it is in families with a BRCA1 mutation. Analyses of BRCA2 mutation data have provided evidence that the risks of breast and ovarian cancer are related to the position of the mutation: truncating mutations in families with the highest risk of ovarian cancer relative to breast cancer are clustered in a region in the middle of exon 11 (Gayther et al. 1997).

The aims of this collaborative study were twofold: first, to estimate the respective proportions of different types of high-risk cancer families in which the disease is due to BRCA1 and BRCA2 and to determine what proportion of families might be due to unidentified genes; and, second, to estimate the penetrance of BRCA2 in a large data set. Groups in the Breast Cancer Linkage Consortium (BCLC) were asked to submit linkage data on markers flanking BRCA1 and BRCA2, for all families containing at least four cases of either female breast cancer diagnosed at age <60 years or male breast cancer diagnosed at any age. Information on BRCA1 mutation testing, including method and outcome, was collected on all families, and mutation details for families with positive mutation tests for BRCA2 were provided. We report here on the results of analyses of 237 families.

Families and Methods

Families

Families included in the study were all those that had been typed, by the Breast Cancer Linkage Consortium (BCLC), for linkage to BRCA1 and that contained at least four cases of either female breast cancer diagnosed at age <60 years or male breast cancer diagnosed at any age, irrespective of any ovarian cancers in the family. Two hundred thirty-seven families were included, 121 (51%) of which had been included in previous BCLC studies. Sixty-seven of the 94 families with at least one case of ovarian cancer but no male breast cancer (referred to subsequently as "breast-ovarian families") had been included in a BCLC study reported by Narod et al. (1995*b*). Twelve of the 26 families with at least one case of male breast cancer were part of a study by Stratton et al. (1994). Only 42 of the 117 families with no ovarian or male breast cancer were in the first BCLC study, which examined linkage to BRCA1 (Easton et al. 1993).

One hundred eighty of the families had been tested for a BRCA1 mutation, including 67 that had been tested by direct sequencing. Mutations within the BRCA1 coding sequence had been reported in 64 families. Thirty-six families were known to have a BRCA2 mutation.

Genetic Markers

All families included in this study had been typed for markers flanking BRCA1. For the majority of families, D17S579 (Hall et al. 1992) and D17S250 (Weber et al. 1990) typings were available. For a small number of families, data were unavailable for these two markers, and data for other markers in the region—in particular, THRA1 (Bowcock et al. 1993) and D17S855 (Gyapay et al. 1994)—were used. The 17q marker typings had not been scored consistently across the families, so, in the analysis, each marker was coded as a system of equally frequent alleles (with the number of alleles chosen to reflect the observed polymorphism of the marker).

One hundred seventy-seven families had, in addition, been typed for markers D13S260 and D13S267, which flank BRCA2 (Wooster et al. 1994). Thirty-five families that had not been typed for linkage to BRCA2 had a mutation in the BRCA1 gene. An additional six families showed strong evidence for linkage to BRCA1 (LOD score >1.0). The remaining 19 families had not been studied for linkage to BRCA2, either because the investigating group concerned had no remaining DNA from critical family members or because it was not feasible because of other reasons. 13q marker typings were scored consistently across families, and the allele frequencies used for analysis were taken from the Genome Database, in which frequencies are based on 56 CEPH chromosomes.

Statistical Methods

Estimating the proportions of families in which the disease is due to BRCA1 and BRCA2.—The proportions of families with disease due to BRCA1 and BRCA2 were estimated by computation of heterogeneity LOD scores. In our first analysis, we assumed that the same breast cancer risks were conferred by mutations in BRCA1, BRCA2, and other susceptibility genes. These risks were based on a "standard" genetic model for breast cancer, derived by Claus et al. (1991), from the Cancer and Steroid Hormone Study (the "CASH" model). Under this model, susceptibility to breast cancer in females is conferred by an autosomal dominant gene with population frequency .003, and the risk of breast cancer at age <70 years is 67% in carriers. The penetrance for ovarian cancer in carriers of the susceptibility gene was derived by assuming a constant relative risk with age and an estimated cumulative risk of 10% by age 60 years (Claus et al. 1993).

In our second analysis, BRCA1 was assumed to confer a penetrance higher than that in the CASH model, as had been suggested by previous studies: a cumulative breast cancer risk in females of 49% by age 50 years and 71% by age 70 years and a cumulative ovarian cancer risk of 16% by age 50 years and 42% by age 70 years (Easton et al. 1993; Narod et al. 1995*b*). BRCA2 and any other genes were still assumed to confer the CASH risks. In the present analysis, we estimate the relative frequency of mutations in BRCA1, BRCA2, and other genes and use these estimates to derive the proportions of families in which the disease is linked to each gene. Computational details are given in Appendix A.

A small number of families ($n = 37$) were not typed for BRCA2 markers, despite having LOD scores <1.0 at BRCA1, and, in some cases ($n = 18$), this was related to having a BRCA1 mutation. This could lead to some bias in the estimates derived by the methods described above, which do not incorporate BRCA1 mutation status. We therefore performed some further analyses, in which the BRCA1 mutation status, as well as the linkage data, were used to estimate the proportion of families in which the disease is due to BRCA1 and BRCA2. In order to do this, an extra parameter, δ, was introduced, to model the probability of identifying a BRCA1 mutation when one existed (see Appendix B). The sensitivity parameter δ is likely to depend on the method used for detection of mutations. The method of detection here refers to the method used initially to screen the coding sequence and splice sites for alterations; all investigating groups used direct sequencing to identify the precise mutation in individuals with screening abnormalities. All groups except two (CRC and ICRF, where the testing was split between more than one laboratory) used one technique consistently. We first estimated the sensitivity of completely sequencing the coding sequence of the BRCA1 gene (Miki et al. 1994) of at least one affected individual (δ_1) and the sensitivity of the other genomic screening techniques taken together (δ_2)—namely, constant-denaturating gel electrophoresis (CDGE; Stoppa-Lyonnet et al. 1997), confirmation-sensitive gel electrophoresis (CSGE; Ganguly et al. 1993), direct screening for deletions and inversions, in combination with protein-truncation analysis (DSDI/PTT; Hogervorst et al. 1995; Peelen et al. 1997), SSCP-heteroduplex analysis (SSCP-HA; Gayther et al. 1996), and SSCP alone. Since δ_1 and δ_2 were estimated identically, subsequent analyses were based on a single estimate for δ, to represent the sensitivity of mutational analysis by any of these methods.

Estimating the penetrance of BRCA2.—The basic method used for estimation of the age-specific risks conferred by BRCA2 was to maximize the LOD score over different values of the penetrance function (Easton et al. 1993). This method is equivalent to maximizing the likelihood conditional on all phenotypic data and hence is free from the ascertainment bias caused by family selection on the basis of multiple affected individuals. In the first analysis, we used the families with an identified mutation in BRCA2. We then repeated the analyses, using all families with six or more cases of either female breast cancer at age 60 years or male breast cancer at any age, assuming that all these families were due to either BRCA1 or BRCA2. The BRCA1 risks were held fixed at the values estimated elsewhere (Easton et al. 1993; Narod et al. 1995*b*). In addition to the BRCA2 risks, an extra parameter was estimated, corresponding to the proportion of all high-risk mutations that are BRCA1 mutations. Further details are given in Appendix C.

Table 1

Heterogeneity Analysis under the CASH Model

Family Group	No. of Families	Proportion (95% CI) of Families with Disease Linked to: BRCA1	BRCA2	Other	LOD Score
All families:					
Four or five breast cancers, male or female	154	.55 (.40–.70)	.12 (.00–.29)	.33 (.14–.53)	19.28
Six or more breast cancers	83	.46 (.32–.60)	.50 (.35–.65)	.04 (.00–.17)	67.44
Overall	237	.52 (.42–.63)	.35 (.24–.46)	.13 (.03–.25)	84.08
All families, no males:					
Four or five breast cancers	140	.61 (.44–.76)	.08 (.00–.25)	.31 (.12–.53)	19.90
Six or more breast cancers	71	.50 (.34–.65)	.44 (.28–.61)	.06 (.00–.21)	50.40
Overall	211	.57 (.45–.68)	.28 (.18–.40)	.15 (.04–.28)	68.13
Male breast cancer	26	.19 (.01–.47)	.77 (.43–.97)	.04 (.00–.42)	18.03
Breast-ovarian cancer, no males:					
One ovarian cancer	42	.69 (.46–.89)	.19 (.04–.41)	.12 (.00–.33)	15.11
Two or more ovarian cancers	52	.88 (.69–.98)	.12 (.02–.31)	.00 (.00–.15)	44.07
Overall	94	.80 (.66–.92)	.15 (.05–.28)	.05 (.00–.17)	58.57
Female breast cancer only:					
Four or five breast cancers	83	.32 (.10–.55)	.09 (.00–.35)	.59 (.26–.89)	2.29
Six or more breast cancers	34	.19 (.04–.41)	.66 (.39–.88)	.15 (.00–.44)	14.18
Overall	117	.28 (.13–.45)	.37 (.20–.56)	.35 (.14–.57)	14.47

Results

Proportions of Families with Disease Due to BRCA1 and BRCA2

Tables 1 and 2 summarize the results of the heterogeneity analyses using BRCA 1 and BRCA2 linkage data only. In table 1 the estimated proportions of families linked to each gene are given, with the CASH model being assumed for all genes. Overall, an estimated 52% of families have disease due to BRCA1, and 35% have disease due to BRCA2. There is significant evidence of families with disease not linked to either gene (χ^2_1 = 8.57; P = .003), although the estimated proportion of families with disease due to other genes is only 13%. In families with fewer than six cases of breast cancer, the proportion of families with disease due to other genes (33%) is higher than it is in families with six or more cases (4%), in which the evidence for other genes is, in fact, not significant. In the majority (77%) of families with one or more cases of male breast cancer, the disease is estimated to be due to BRCA2, with a smaller proportion (19%) estimated as being due to BRCA1. The evidence for other susceptibility genes is not significant, although the upper confidence limit on the remaining proportion of families is 42%. Of the families with breast and ovarian cancer but with no male case, the large majority of families are estimated to have disease due to BRCA1 (80%), with a smaller proportion (15%) estimated to be due to BRCA2. The evidence for other genes is weak in this group of families, the upper confidence limit for the proportion of disease due to other genes being 17%. Among the 117 families with female breast cancer only, the proportions of families estimated to have disease due to BRCA1, BRCA2, and other genes were similar (28%, 37%, and 35%, respectively). The estimated proportion of families with disease due to other genes was higher in families with fewer than six cases (59%) than in families with six or more cases (15%, with a lower confidence limit of 0%). Interestingly, in families with six or more cases of breast cancer and no ovarian cancer, the largest proportion of disease was due to BRCA2 (66%, compared with 19% due to BRCA1).

In table 2 the estimated proportions of high- risk mutations, as well as the corresponding estimated proportions of families, attributable to each gene are given, under the assumption that BRCA1 confers the risks estimated in previous consortium analyses whereas BRCA2 and other genes confer risks given by the CASH model. Families containing male breast cancers have been excluded from table 2, since (1) the penetrance in males is uncertain for all genes and (2) estimation of the proportion of high-risk mutations attributable to each gene is strongly dependent on the assumed penetrances. In general, the estimated proportions of families with disease due to each of the genes is very similar in the two analyses, strengthening the support for these estimates. The largest difference is in the families with six or more cases of female breast cancer only, where the estimated proportion due to BRCA1 is higher (29% vs. 19%) and the proportion due to other genes is, correspondingly, lower (5% vs. 15%), when a higher penetrance for BRCA1 is assumed. There is stronger evidence for other predisposition genes in the breast-ovarian can-

Am. J. Hum. Genet. 62:676–689, 1998

Table 2

Heterogeneity Analysis Allowing BRCA1 to Confer Higher Penetrance

Family Group	No. of Families	Proportion (95% CI) of High-Risk Mutations and Families[a]			LOD Score
		BRCA1	BRCA2	Other	
All families, no males:					
Four or five breast cancers	140	.52 (.31–.73)	.06 (.00–.24)	.42 (.19–.67)	23.04
		.56 (.42–.70)	.05 (.00–.19)	.39 (.21–.56)	
Six or more breast cancers	71	.39 (.22–.58)	.51 (.32–.70)	.10 (.00–.28)	55.65
		.50 (.45–.55)	.42 (.34–.49)	.08 (.00–.17)	
Overall	211	.47 (.33–.62)	.31 (.18–.45)	.22 (.09–.38)	76.11
		.54 (.46–.61)	.27 (.19–.35)	.19 (.10–.30)	
Breast-ovarian cancer, no males:					
One ovarian cancer	42	.60 (.31–.86)	.20 (.04–.47)	.20 (.03–.47)	13.58
		.67 (.52–.81)	.16 (.07–.30)	.17 (.05–.30)	
Two or more ovarian cancers	52	.42 (.19–.77)	.52 (.10–.81)	.06 (.00–.52)	46.73
		.80 (.72–.89)	.18 (.05–.27)	.02 (.00–.15)	
Overall	94	.52 (.31–.76)	.29 (.10–.52)	.19 (.03–.42)	59.99
		.74 (.66–.83)	.16 (.08–.24)	.10 (.03–.19)	
Female breast cancer only:					
Four or five breast cancers	83	.43 (.15–.69)	.07 (.00–.30)	.50 (.20–.84)	2.75
		.36 (.16–.55)	.08 (.00–.28)	.56 (.28–.83)	
Six or more breast cancers	34	.35 (.11–.63)	.61 (.33–.85)	.04 (.00–.32)	15.66
		.29 (.19–.38)	.66 (.52–.76)	.05 (.00–.25)	
Overall	117	.42 (.23–.62)	.32 (.16–.49)	.26 (.07–.48)	16.21
		.35 (.24–.47)	.36 (.22–.48)	.29 (.10–.47)	

[a] Within each table cell, the underlined entry is the proportion of high-risk mutations, and the entry below it is the proportion of families.

cer families in this analysis, although the estimated proportion is still small (10%), and, in families with at least two cases of ovarian cancer, there is still no significant evidence of other predisposition genes.

The proportions of high-risk mutations that occur in each gene should not depend on family type, so the estimates should be consistent across subgroups of families, providing that the penetrances of the genes are well estimated. If the families with male cases are excluded, an estimated 47% of high-risk mutations occur in BRCA1 and 31% occur in BRCA2, leaving 22% of mutations in unidentified genes. The estimates do not differ significantly between the breast-ovarian cancer families and the families with female breast cancer only (χ^2_2 =; P = .81) or between families with only one ovarian cancer and families with two or more ovarian cancers (χ^2_2 = 1.47; P = .48). Estimates do differ, however, between families with fewer than six cases of female breast cancer and families with six or more cases (χ^2_2 = 11.88; P = .003), reflecting the difference seen in the families without ovarian cancer, in which 7% of mutations are attributed to BRCA2 and 50% are attributed to other genes if there are fewer than six cases in the family, compared with 61% and 4%, respectively, if there are six or more cases (χ^2_2 = 10.13; P = .006).

Analysis Including BRCA1 Mutation Data

One hundred eighty of the families were tested for mutations in BRCA1, the majority by direct sequencing (67 families) or by one or more of the following: CDGE, CSGE, DSDI/PTT, SSCP-HA, and SSCP alone (106 families). One family had been tested by PTT only, and six families had been analyzed by SSCP across a portion of the gene.

In the initial heterogeneity analyses incorporating BRCA1 testing information, the estimated proportion of mutations identified by direct sequencing was allowed to differ from the proportion identified by CDGE, CSGE, DSDI/PTT, SSCP-HA, or SSCP. Both the family tested by PTT and the six families only partially analyzed were assumed not to have been tested. Across all families, the estimated sensitivity of sequencing under the CASH model was .64 (95% CI .45–.84), which was the same as the estimated sensitivity for the other methods (.64 [95% CI .49–.80]). The estimated proportions of families with disease due to BRCA1 (52% [95% CI 42%–62%]), BRCA2 (32% [95% CI 22%–43%]), and other genes (16% [95% CI 6%–28%]) were almost identical to those obtained by use of linkage data only (see table 1). We subsequently reanalyzed the data, assuming a single sensitivity parameter for all the standard screening methods.

Table 3

Heterogeneity Analysis under the CASH Model Incorporating BRCA1 Mutation Data

Family Group	No. of Families			Proportion (95% CI) of Linked Families			Proportion (95% CI) of Mutations Identified[a]
	Total	Tested for BRCA1	Mutation Positive	BRCA1	BRCA2	Other	
All families:							
Four or five breast cancers, male or female	154	121	40	.50 (.36–.65)	.13 (.0–.28)	.37 (.19–.56)	.64 (.46–.84)
Six or more breast cancers, male or female	83	59	24	.50 (.37–.63)	.45 (.31–.58)	.05 (.00–.17)	.69 (.50–.86)
Overall	237	180	64	.52 (.42–.62)	.32 (.22–.43)	.16 (.06–.28)	.63 (.51–.77)
All families, no males:							
Four or five breast cancers	140	114	39	.54 (.40–.69)	.08 (.00–.23)	.38 (.19–.58)	.63 (.45–.82)
Six or more breast cancers	71	52	23	.55 (.41–.69)	.38 (.24–.53)	.07 (.00–.21)	.70 (.51–.87)
Overall	211	166	62	.55 (.45–.66)	.26 (.16–.37)	.19 (.08–.31)	.64 (.51–.77)
Male breast cancer	26	14	2	.16 (.02–.48)	.76 (.43–.97)	.08 (.00–.44)	.67 (.09–1.0)
Breast-ovarian cancer, no males:							
One ovarian cancer	42	39	20	.69 (.50–.86)	.21 (.06–.40)	.10 (.00–.28)	.79 (.56–.96)
Two or more ovarian cancers	52	45	26	.91 (.76–.99)	.09 (.01–.24)	.00 (.00–.11)	.62 (.46–.77)
Overall	94	84	46	.81 (.68–.91)	.14 (.05–.26)	.05 (.00–.16)	.68 (.55–.81)
Female breast cancer only:							
Four or five breast cancers	83	63	11	.28 (.11–.50)	.05 (.00–.29)	.67 (.35–.89)	.61 (.29–1.0)
Six or more breast cancers	34	19	5	.21 (.08–.42)	.60 (.34–.83)	.19 (.01–.45)	1.0 (.41–1.0)
Overall	117	82	16	.26 (.13–.42)	.32 (.17–.50)	.42 (.21–.62)	.68 (.38–1.0)

[a] By sequencing, CDGE, CSGE, DSDI/PTT, SSCP-HA, SSCP, or PTT.

Table 3 describes the heterogeneity results under the CASH model with the incorporation of the BRCA1 mutation data. The proportion of mutations identified refers here to a single estimate for all methods. PTT alone (which had been used in one family) was assumed to be as sensitive as sequencing, CDGE, CSGE, DSDI/PTT, SSCP-HA, or SSCP, and the partial screening by SSCP, which was used in six families, was assumed to be 25% as efficient as the other methods. The results in table 3 show that, when mutation data are incorporated, the estimated proportions of families with linkage to each gene are almost identical to those estimated from linkage data alone. The estimated proportions of mutations being identified is consistent, in all family types, with the overall estimate of 63% (95% CI 51%–77%). We also repeated the analyses under the alternative model, allowing the risks conferred by BRCA1 to be higher than those conferred by BRCA2 or the other genes; again, incorporating the mutation data had no influence on the heterogeneity estimates (data not shown).

Penetrance of BRCA2

The overall penetrance of BRCA2 was estimated in the 32 families in which a BRCA2 mutation was found and in which D13S267 typing was available and informative. Table 4 gives the risks of breast or ovarian cancer in these families, obtained by maximizing the LOD score over the age-specific incidence rates of disease. The estimated overall penetrance is 29% (95% CI 9%–44%) by age 50 years and 88% (95% CI 48%–97%) by age 70 years. Most of this risk is due to breast cancer; among mutation carriers, there are no cases of ovarian cancer at age <40 years and only two cases of ovarian cancer at age <50 years. The estimated breast cancer risk in this group is 28% (95% CI 9%–44%) by age 50 years and 84% (95% CI 43%–95%) by age 70 years. The corresponding ovarian cancer risks are 0.4% (95% CI 0%–1%) by age 50 years and 27% (95% CI 0%–47%) by age 70 years.

Table 5 shows the risks obtained for BRCA2 mutation carriers if families with at least six cases of breast cancer (females at age <60 years or males at any age) are attributed to BRCA1 or BRCA2. (One family was excluded from this analysis, because of a consanguineous relationship that could not be ignored.) In the age group 40–59 years, the estimated overall risks and breast cancer risks are higher than the risks in the mutation families, although the differences do not reach statistical significance (difference in overall cancer risks by age 50 years [$P = .11$]; difference by age 60 years [$P = .50$]), and the risks by age 70 years are very similar in the two analyses (86% overall vs. 88% overall). The risks previously estimated for BRCA1 are also given, for comparison. The estimated risks for BRCA2 are slightly lower by ages 40 years and 50 years and are slightly higher by age 70 years, but they are not significantly different at any age. When only the mutation families are considered, the estimated BRCA2 risk, up to age 50 years, is more markedly lower than is the BRCA1 risk, although, again, the risks by age 70 years are similar.

Table 4

Incidence and Cumulative Risks of Breast and Ovarian Cancer in BRCA2 Mutation Carriers

Age Group (Years)	Breast and Ovarian Cancer		Breast Cancer Only			Ovarian Cancer Only		
	Incidence	Cumulative Risk (95% CI)	No.	Incidence	Cumulative Risk (95% CI)	No.	Incidence	Cumulative Risk (95% CI)
20–29	.000633	.006 (0–.019)	7	.000633	.006 (0–.019)	0	.00	.00
30–39	.0118	.12 (0–.24)	61	.0118	.12 (0–.24)	0	.00	.00
40–49	.0215	.29 (.090–.44)	99	.0210	.28 (.090–.44)	2	.000425	.004 (0–.011)
50–59	.0390	.52 (.24–.70)	44	.0318	.48 (.22–.65)	10	.00722	.074 (0–.15)
60–69	.142	.88 (.48–.97)	20	.118	.84 (.43–.95)	4	.0236	.27 (0–.47)

NOTE.—All data are for first cancers: the number exclude second primaries and known noncarriers of mutation or "linked"-haplotype.

Table 6 provides the risks for the BRCA2 mutation families, according to mutation location. The gene has been divided into three regions, the middle region being the ovarian cancer–cluster region (OCCR) suggested by Gayther et al. (1997). One family with a missense mutation was excluded from this analysis. The estimated breast cancer risks appear to be similar in all three groups. The estimated ovarian cancer risk is, however, higher in the OCCR, in agreement with the results of Gayther et al. (1997), although the confidence limits are very wide.

Discussion

This report describes the analysis of the largest collection of breast cancer families in the world, for linkage to BRCA1 and BRCA2. Two hundred thirty-seven families with four or more cases of either female breast cancer diagnosed at age <60 years or male breast cancer at any age were contributed by 21 investigating groups from nine countries. All families were typed with genetic markers flanking BRCA1, and 177 families were typed with genetic markers flanking BRCA2.

As had been suggested by other BCLC studies, almost all breast-ovarian cancer families appear to be compatible with linkage to BRCA1 or BRCA2 (Narod et al. 1995*a*, 1995*b*). Only 2 families (CJP3 and MDC60) of the 94 breast-ovarian cancer families had multipoint LOD scores of <-1.0 at both loci; both these families had been screened for germ-line BRCA1 mutations, although neither was sequenced, and neither had been tested for a mutation in BRCA2. Each of these two families had only one case of ovarian cancer. One of these families (MDC60) recently has been shown, tentatively, to have disease linked to markers on chromosome 8p (Seitz et al. 1997), a location for a breast cancer–susceptibility gene first suggested by Kerangueven et al. (1995). There was very little evidence for another predisposition gene in the 52 families containing two or more cases of ovarian cancer; under the CASH model the point estimate for the proportion of remaining families was 0.0, and under the model allowing BRCA1 to confer a higher penetrance it was 2%.

This study has confirmed that the large majority of families containing both male and female breast cancer have disease that is due to BRCA2. In slight contrast to previous linkage studies (Stratton et al. 1994), there is

Table 5

Estimated Penetrance of BRCA2 and Comparison with Penetrance of BRCA1

Age (Years)	Penetrance (95% CI)					
	BRCA2					
	32 Families with BRCA2 Mutation		82 Families with Six or More Breast Cancers[b]		BRCA1[a]	
	Breast and Ovarian —Cancer	Breast Cancer —Only	Breast and Ovarian —Cancer	Breast Cancer —Only	Breast and Ovarian —Cancer	Breast Cancer —Only
30	.006 (0–.019)	.006 (0–.19)	.046 (.004–.086)	.046 (.004–.086)	.036 (0–.14)	.036 (.0–.14)
40	.12 (0–.24)	.12 (0–.24)	.12 (.048–.18)	.12 (.048–.18)	.18 (0–.36)	.18 (.0–.35)
50	.29 (.090–.44)	.28 (.090–.44)	.46 (.31–.57)	.45 (.31–.57)	.57 (.33–.73)	.49 (.28–.64)
60	.52 (.24–.70)	.18 (.22–.65)	.61 (.39–.76)	.59 (.37–.73)	.75 (.53–.87)	.64 (.43–.77)
70	.88 (.48–.97)	.84 (.43–.95)	.86 (.25–.98)	.83 (.27–.96)	.83 (.65–.92)	.71 (.53–.82)

[a] Source: Narod et al. (1995*b*).

[b] Proportion of BRCA2 mutations is .84 (95% CI .56–1.0) when it is assumed that BRCA1 and BRCA2 account for cancers in all families. Corresponding proportion of families with BRCA2 mutations is .50 (95% CI .39–1.0).

Table 6

Cumulative Risks of Breast and Ovarian Cancer in BRCA2 Mutation Carriers, by Mutation Location

	Cumulative Risk (95% CI)					
	Exons 1–10 and 11, Nucleotides 1–1034		Exon 11, Nucleotides 1035–6629		Exon 11, Nucleotides 6630–end and Exons 12–23	
Age	Breast Cancer	Ovarian Cancer	Breast Cancer	Ovarian Cancer	Breast Cancer	Ovarian Cancer
30	.0011 (0–.0042)	0	.016 (0–.10)	0	.017 (0–.071)	0
40	.14 (0–.34)	0	.090 (0–.30)	0	.10 (0–.25)	0
50	.26 (0–.47)	.0036 (0–.12)	.27 (0–.62)	0	.32 (0–.56)	.0063 (0–.021)
60	.41 (.053–.63)	.053 (0–.13)	.47 (0–.82)	.24 (0–.64)	.60 (.12–.82)	.052 (0–.13)
70	.82 (0–.97)	.26 (0–.53)	.83 (0–.99)	.76 (0–.98)	.78 (0–.97)	.052 (0–13)

now clear evidence, both from linkage data and from mutation data (two families had identified mutations), that a proportion of these families are the result of BRCA1 mutations; 16% (95% CI 2%–48%) of families with a male case were estimated to be due to BRCA1. Of the seven families with two or more cases of male breast cancer, four had BRCA2 mutations, and data for one additional family were consistent with linkage to BRCA2 (LOD score 0.8), whereas data for the remaining two families were consistent with linkage to BRCA1 but not to BRCA2. The most likely interpretation of these findings is that BRCA1 mutations do confer an increased risk of male breast cancer but that the risk is lower than the risk conferred by BRCA2.

Heterogeneity results for the families containing only female breast cancer are of particular interest because the majority of families in the population are of this type, and the BCLC data on these families have expanded enormously since our first study (Easton et al. 1993). In the current study there were 117 families with no ovarian or male breast cancers. We estimate that in ~60% of these families the disease is likely to be due to a BRCA1 mutation or a BRCA2 mutation, regardless of the analysis model used. Of the families with six or more cases of female breast cancer, 21% are estimated to have disease that is due to BRCA1, and 60% are estimated to have disease that is due to BRCA2, leaving 19% due to other genes, when the CASH model is assumed for all genes and the BRCA1 mutation data are incorporated, with similar estimates in the other analyses. There were no large families with only female breast cancer that had LOD scores <-1.0 at both BRCA1 and BRCA2. In the group of families with four or five cases of female breast cancer, the proportions of families with disease attributable to BRCA1, BRCA2, and other genes are 28%, 5%, and 67%, respectively. The low proportion of families with four or five cases of female breast cancer that is due to BRCA2, as compared with the proportion of families with six or more cases due to it, may be due partly to chance, particularly since the latter group contains only 34 families. Both the low estimate of the proportion of families with only breast cancer due to BRCA1 and the slightly lower estimate for the proportion of the larger families with only breast cancer due to it are likely to be due to the significant ovarian cancer risk associated with BRCA1, although it is worth noting that a BRCA1 mutation was identified in 5 of the 34 families with six or more breast cancers and no ovarian cancer. The most important conclusion from this analysis is that a large proportion, perhaps the majority, of families with five or fewer cases of breast cancer and no ovarian or male breast cancer cases are not due to either BRCA1 or BRCA2. This is also indicated by the recent study by Serova et al. (1997), who performed mutation screening of BRCA1 and BRCA2 in 31 site-specific breast cancer families (7 of which have been included in the present study) and found mutations in only 8 of them. Similar results also were observed by Håkansson et al. (1997) and Schubert et al. (1997). A particular value of the current study, however, in addition to its much larger size, is that its results are based primarily on linkage and hence are not dependent on the sensitivity of the mutation testing. The fact that the proportion of families without linkage is much larger among families with fewer than six cases is consistent with the hypothesis that susceptibility alleles in other breast cancer genes confer risks lower than those conferred by BRCA1 or BRCA2 but are, correspondingly, more common in the population. The other known susceptibility genes—such as TP53, ATM, the mismatch-repair genes, the newly identified PTEN gene involved in Cowden syndrome (Liaw et al. 1997), and the gene(s) responsible for Peutz-Jeghers syndrome (Hemminki et al. 1997)—are unlikely to explain the high frequency of disease in an important fraction of these families. We conclude that other susceptibility genes responsible for a large fraction of familial breast cancer remain to be identified. Note that the present study did not examine linkage or mutation data in families with fewer than four cancer cases, so we cannot make any direct estimate of the contribution of BRCA1 and BRCA2 to families with two or three cases (in practice, by far the largest group), but, by extrapolation from the results in this

study, one would predict that their contribution would be relatively minor.

Overall, the heterogeneity analyses suggest that, in the western European and North American populations studied, BRCA1 is a slightly more common cause of cancer in high-risk families than is BRCA2. There may, however, be important local variations due to founder effects, most notably in Iceland, where, in six of the eight high-risk families, the disease is due to a single BRCA2 mutation. Moreover, the results do not imply that BRCA1 mutations are more common in lower-risk families, breast cancer cases, or the general population. In fact, if the penetrance of BRCA2 were markedly lower at younger ages, BRCA2 mutations could be more common in the population.

Although this was primarily a study based on linkage rather than on mutation data, we also performed some heterogeneity analyses incorporating BRCA1 mutation data. The main aim of these additional analyses was to ensure that no serious biases had been introduced by selective typing of BRCA2 markers on the basis of BRCA1 mutation status. Intuitively, such bias seem unlikely, since only a small proportion of data could have been influenced, and the analyses incorporating the mutation data obtained essentially the same estimates as were obtained by the analyses without mutation data. As a by-product of these analyses, we were able to obtain an estimate of the sensitivity of the mutation testing used in this study. Inevitably, this is a quite crude analysis, since different investigating groups used different screening techniques. Assuming that all methods (other than screening less than the whole of the gene) were equally sensitive, we estimated an identification probability of 63% (95% CI 51%–77%). In this data set there was no evidence that sensitivity differed between sequencing and other methods. The obvious explanation for this relatively low sensitivity estimate, even for direct sequencing, is that a substantial fraction of BRCA1 alterations occur outside the coding sequence and splice sites. This explanation is supported by the observation that no mutation has been identified for a relatively high proportion of families where the disease is clearly linked to BRCA1. Of the 33 families with a LOD score >1.0 for linkage to BRCA1, 30 had been screened for a BRCA1 mutation. On the basis of the posterior probabilities in these families, one would predict that 29.3 (98%) of cases should be due to BRCA1. In fact, 21 mutations (70%) were found (3 of 6 tested by direct sequencing, 12 of 17 tested by CDGE, CSGE, DSDI/PTT, or SSCP-HA, and 6 of 7 tested by SSCP alone). Of the nine families without mutations, two (UTAH 2035 and IARC 2090) were shown to have loss of the entire BRCA1 transcript in cDNA, and one (IARC 1816) was found to have loss of exon 18 in transcript, but with no splice-site alteration. These families are therefore false negatives, according to the usual DNA-based testing methods. The true proportion of families with inferred regulatory mutations may be much higher, since cDNA testing is not always possible or informative. Other possible explanations for the "missing" mutations would include large deletions, promoter-sequence alterations, or simply a coding-sequence alteration that was missed.

We were able to estimate the penetrance of BRCA2 in families with a known mutation and to provide evidence for a high lifetime risk of breast or ovarian cancer: our overall estimates were 29% (95% CI 9%–44%) by age 50 years and 88% (95% CI 48%–97%) by age 70 years, the majority of which is attributable to breast cancer. Although not significantly different, the estimated breast cancer risk at younger ages was somewhat lower in BRCA2 mutation carriers compared with BRCA1 mutation carriers; 30% (76 of 250) of breast cancers at age <70 years in the 36 families with a germline BRCA2 mutation were diagnosed at age <40 years and 73% (182 of 250) were diagnosed at age <50 years, compared with 50% (210 of 416) diagnosed at age <40 years and 80% (333 of 416) diagnosed at age <50 years in the 64 families with a germ-line BRCA1 mutation. The overall risks of ovarian cancer (7% by age 60 years and 27% by age 70 years) are lower than the corresponding estimates for BRCA1 that had been obtained by previous consortium analyses (31% by age 60 and 42% by age 70 years [Easton et al. 1993; Narod et al. 1995*b*]; and 30% by age 60 years and 63% by age 70 years [Easton et al. 1995]), although they still are substantially elevated above the risks in the general population. This is entirely consistent with the heterogeneity results showing that BRCA2 is responsible for a smaller—but still significant—fraction of breast-ovarian cancer families than is due to BRCA1.

Our penetrance estimates assumed that all mutations conferred the same risks, which is likely to be an oversimplification. Gayther et al. (1997) found evidence for an increased risk of ovarian cancer, relative to the risk of breast cancer, associated with mutations in the central portion of BRCA2 (the OCCR), compared with mutations outside this region. Our study provides some support for this genotype-phenotype correlation, in that higher risks of ovarian cancer—but similar risks of breast cancer—were estimated for BRCA2 mutations in the OCCR, compared with mutations outside it.

Recently Struewing et al. (1997) estimated the penetrance of BRCA1 and BRCA2 mutations on the basis of the family histories of 120 Ashkenazi Jewish volunteers found to have any of the three founder mutations common in this population. In carriers of the 6174delT mutation, which lies within the OCCR, they estimated a breast cancer risk of ~50% by age 70 years and an ovarian cancer risk of ~10% by age 60 years and 18% by age 70 years. These estimates are somewhat lower

than the estimates derived in the present study, but they are not inconsistent, given the wide confidence limits.

In this study, we have not attempted to estimate the risk of male breast cancer in BRCA2 families. The maximum-LOD-score approach would not provide a precise estimate, because the penetrance is clearly quite low. Using the two largest BRCA2 families with linkage (families UTAH 107 and CRC 186) Easton et al. (1997) estimated the cumulative risk of male breast cancer in BRCA2 carriers to be 6% by age 70 years, but this was based on only four observed cases and hence is very imprecise. There is also circumstantial evidence of variation in male breast cancer risk; whereas the large majority (19 of 26) of "male breast cancer" families in this study contain only one male case, two families contained four cases each. In both of these families, BRCA2 mutations have been identified. This variation in risk is likely to be due, at least in part, to modifying factors, since both families have mutations identical to those in large breast cancer families that do not have male breast cancer cases.

In addition to the risks of breast and ovarian cancer, previous studies have suggested an increased risk of a number of other cancers in BRCA2 carriers, including prostate cancer (Tulinius et al. 1992), pancreatic (Phelan et al. 1996), and ocular melanoma (Easton et al. 1997). The risks of other cancers in the collaborative BCLC data set are currently being evaluated.

The penetrance estimates provided by this study, together with the comparable estimates for BRCA1 carriers, should be useful for genetic counseling of mutation carriers. Such estimates must always be used with caution, however. They are appropriate for counseling in multiple-case families but may not apply to mutation carriers with little or no family history. The risks to such individuals could be lower than those estimated here, either if there is risk variation between mutations, or if modifying genes or other familial risk factors strongly influence penetrance.

Acknowledgments

The analysis of these data was funded by the Cancer Research Campaign. Meetings of the Breast Cancer Linkage Consortium, as well as data management, were supported by EC Concerted Action. Data collection and laboratory analysis were supported by the Cancer Research Campaign, the Imperial Cancer Research Fund, Aberdeen Royal Hospitals Endowment, NIH grant CA55914, U.S. Army grants DAMD-17-94-J-4260 and DAMD-17-94-J-4340, the ARC, Ligue Contre le Cancer, Fédération Nationale des Centres de Lutte Contre le Cancer, Fédération des Groupements d'Entreprises dans la Lutte Contre le Cancer, INSERM CR 4U00 3C, Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie grant 01ZZ9509, Deutsche Krebshilfe, and Swiss Cancer League grants AKT 332 and AKT 463. We thank Lesley McGuffog and Amy Storfer-Isser for their help with the statistical analysis, and we thank François Eisinger and Daniel Birnbaum for help with data collection and laboratory work.

Appendix A

Heterogeneity Analysis

Linkage analyses were performed by means of the FASTLINK version of the LINKAGE program (Lathrop et al. 1984; Cottingham et al. 1993). The CASH model was modified to assume that the incidence of breast cancer in gene carriers age ≥ 70 years was the same as in carriers age 60–69 years. The risks in noncarrier females were assumed to follow population rates for breast and ovarian cancer for England and Wales. (All families were from western European or North American populations that have similar breast and ovarian cancer incidence rates.) For implementation in LINKAGE, 21 liability classes were constructed, allowing for seven age groups and three phenotypes (unaffected, affected with breast cancer, and affected with ovarian cancer). Individuals with both breast and ovarian primary cancers were treated as if they were affected with breast cancer at age <30 years, in order to maximize their probability of being gene carriers. Males with breast cancer were also treated as if they were females affected at age <30 years, because of the rarity of male breast cancer. All unaffected males were treated as they were unaffected females of age <30 years, which is approximately equivalent to being of unknown disease status.

For most families, multipoint LOD scores for linkage to BRCA1 and BRCA2 were computed by use of two flanking markers and the disease. A small minority of families contained consanguineous or marriage loops, and these were broken if this resulted in no loss of information, but, when a loop had to be retained, the most informative marker on each chromosome was used in a two-point analysis. BRCA1 was assumed to lie either midway between THRA1 and D17S579 (1.3 cM from THRA1 on the male genetic map, with a 2:1 female: male genetic-distance ratio) or at D17S855. BRCA2 was assumed to lie 1 cM distal to D13S260 and 2 cM proximal to D13S267.

Estimates of proportions of families with disease due to BRCA1 and BRCA2 were obtained by maximization of the heterogeneity LOD score,

$$\begin{aligned} &\mathrm{LOD}(\alpha_1, \alpha_2) \\ &\quad = \Sigma \log_{10}[\alpha_1 10^{\mathrm{LOD}_1(\underline{\theta}_1)} + \alpha_2 10^{\mathrm{LOD}_2(\underline{\theta}_2)}, \\ &\quad\quad + (1 - \alpha_1 - \alpha_2)] \end{aligned} \tag{A1}$$

with respect to α_1 and α_2, where α_1 and α_2 are the respective proportions of families with disease linked to

BRCA1 and BRCA2. The proportion of families with disease due to other high-penetrance genes is estimated by $1 - \alpha_1 - \alpha_2$. θ_1 is the vector of recombination fractions between the 17q markers and BRCA1, and θ_2 is the vector of recombination fractions between the 13q markers and BRCA2. $LOD_1(\underline{\theta_1})$ and $LOD_2(\underline{\theta_2})$ are the multipoint LOD scores for linkage to BRCA1 and BRCA2, respectively. Summation is over all families.

Maximizing this heterogeneity LOD score provides consistent estimates for α_1 and α_2. This is true despite the fact that families showing strong evidence for linkage to BRCA1 were sometimes not typed for BRCA2 markers. The reasoning behind this is as follows. Equation (A1) may be rewritten as

$$\begin{aligned}
&LOD(\alpha_1, \alpha_2) \\
&\quad = \Sigma \log_{10} L(D, M_1, M_2/\underline{\theta_1}, \underline{\theta_2}, \alpha_1, \alpha_2) \\
&\qquad - \Sigma \log_{10} L(D, M_1, M_2/\underline{1/2}, \underline{1/2}) \\
&\quad = \Sigma \log_{10} L(D, M_1, M_2/D, \underline{\theta_1}, \underline{\theta_2}, \alpha_1, \alpha_2) \\
&\qquad - \Sigma \log_{10} L(M_1, M_2) ,
\end{aligned}$$

where D represents the disease phenotypes and M_1 and M_2 represent the observed marker genotypes. Thus maximizing the LOD score is equivalent to maximizing the likelihood of all marker and disease phenotypes, conditional on the disease phenotypes (Elston 1995). This, in turn, may be written as

$$\begin{aligned}
&LOD(\alpha_1, \alpha_2) \\
&\quad = \Sigma \log_{10} L(D, M_1/D, \underline{\theta_1}, \alpha_1) \\
&\qquad + \Sigma \log_{10} L(D, M_1, M_2/D, M_1 \underline{\theta_1}, \underline{\theta_2} \alpha_1, \alpha_2) \\
&\qquad - \Sigma \log_{10} L(M_1, M_2) .
\end{aligned}$$

The first term is a conditional log likelihood for the markers at the BRCA1 locus, and it therefore gives consistent estimates of α_1. The second term is a conditional log likelihood for the BRCA2 markers, conditional on the disease status and the BRCA1 marker genotypes, which is therefore a valid log likelihood leading to consistent estimates of α_1 and α_2, even though there is some selection based on M_1. The third term is simply a constant. Maximizing LOD (α_1,α_2) will therefore give consistent estimates for α_1 and α_2 (by the usual argument that, since the expected derivative of each term on the right-hand side of the equation is zero at the true values of α_1 and α_2, since each is a valid log likelihood, this must also be true of the sum).

Analyses allowing BRCA1 to confer a higher penetrance (the "BRCA1 model") were based on maximization of the LOD score,

$$\begin{aligned}
&LOD(\alpha_1, \alpha_2) \\
&\quad = \Sigma \log_{10} [\alpha_1 L_1(D, M_1, M_2/\underline{\theta_1}, \underline{1/2}) \\
&\qquad + \alpha_2 L_2(D, M_1, M_2/\underline{1/2}, \underline{\theta_2}) \\
&\qquad + (1 - \alpha_1 - \alpha_2) L_3(D, M_1, M_2/\underline{1/2}, \underline{1/2})] \\
&\qquad - \Sigma \log_{10} [\alpha_1 L_1(D, M_1, M_2/\underline{1/2}, \underline{1/2}) \\
&\qquad + \alpha_2 L_2(D, M_1, M_2/\underline{1/2}, \underline{1/2}) \\
&\qquad + (1 - \alpha_1 - \alpha_2) L_3(D, M_1, M_2/\underline{1/2}, \underline{1/2})] ,
\end{aligned}$$

with respect to α_1, the proportion of BRCA1 mutations as a proportion of all high-risk mutations, and α_2, the proportion of BRCA2 mutations as a proportion of all high-risk mutations, L_1 represents the likelihood for a family under the BRCA1 model, and L_2 represents the likelihood under the CASH model. The relative frequency of mutations in high-risk genes other than BRCA1 and BRCA2 is estimated by $1 - \alpha_1 - \alpha_2$. Summation is over all families.

Dividing the numerator and the denominator by $L_2(D,M_1,M_2/1/2, 1/2)$ allows simplification to

$$\begin{aligned}
&LOD(\alpha_1, \alpha_2) \\
&\quad = \Sigma \log_{10} [\alpha_1 10^{LOD_1(\underline{\theta_1}) - G} \\
&\qquad + \alpha_2 10^{LOD_2(\underline{\theta_2})} + (1 - \alpha_1 - \alpha_2)] \\
&\qquad - \Sigma \log_{10} [\alpha_1 10^{G} + \alpha_2 + (1 - \alpha_1 - \alpha_2)] . \qquad \text{(A2)}
\end{aligned}$$

$LOD_1(\theta)_1$ is the multipoint LOD score for linkage to BRCA1, computed under the BRCA1 model, and $LOD_2(\theta)_2$ is the multipoint LOD score for linkage to BRCA2, computed under the CASH model. $G = log_{10}[L_1(D)/L_2(D)]$; that is, G is the log of the ratio of the likelihood of the disease phenotypes in the family under the BRCA1 model to the likelihood of the disease phenotypes under the CASH model. (For practical purposes, $L_1(D)/L_2(D)$ is the same as $L_1(D,M/1/2)/L_2(D,M/1/2)$; hence, both numerator and denominator may be computed in LINKAGE, with the same marker data but with different models; we used the 17q marker typings for which we had already computed the numerator and denominator.)

α_1, α_2, and $1 - \alpha_1 - \alpha_2$ no longer estimate the proportions of families with disease due to BRCA1, BRCA2, and other genes, since these depend on the genes' penetrances as well as on their frequencies. For each family, the posterior probabilities p_1 (i.e., of segregating a BRCA1 mutation), p_2 (i.e., of segregating a BRCA2 mutation), and p_3 (i.e., of segregating a mutation in another high-risk gene) were therefore computed, where

$$p_1 = \alpha_1 10^{LOD_1(\theta_1)-G}/[\alpha_1 10^{LOD_1(\theta_1)-G} + \alpha_2 10^{LOD_2(\theta_2)} + (1-\alpha_1-\alpha_2)] ,$$

$$p_2 = \alpha_2 10^{LOD_2(\theta_2)}/[\alpha_1 10^{LOD_1(\theta_1)-G} + \alpha_2 10^{LOD_2(\theta_2)} + (1-\alpha_1-\alpha_2)] ,$$

and $p_3 = 1 - p_1 - p_2$. The proportion of families due to each gene was then estimated as the average of the corresponding posterior probabilities.

Appendix B

Heterogeneity Analysis Incorporating BRCA1 Mutation Data

Parameters α_1, α_2, and δ were estimated by joint maximization of a likelihood incorporating both linkage and mutation data. Under the assumption that the CASH model applies to all genes, a family with an identified mutation contributes $\log_{10}(\alpha_1 \delta 10^{LOD_1(\theta_1)})$ to the total likelihood expression, a family in which the mutation has been looked for but was not found contributes

$$\log_{10}[\alpha_1(1-\delta)10^{LOD_1(\theta_1)} + \alpha_2 10^{LOD_2(\theta_2)} + (1-\alpha_1-\alpha_2)] ,$$

and a family in which mutation testing has not been performed contributes as previously described (see eq. [A1].

The parameter δ can similarly be introduced into the analysis, allowing BRCA1 to confer a higher penetrance. A family with an identified mutation then contributes

$$\log_{10}(\alpha_1 \delta 10^{LOD_1(\theta_1)-G}) - \log_{10}[\alpha_1 10^{G} + \alpha_2 + (1-\alpha_1-\alpha_2)]$$

to the total likelihood expression. A family in which the mutation has been looked for but has not been found contributes

$$\log_{10}[\alpha_1(1-\delta)10^{LOD_1(\theta_1)-G} + \alpha_2 10^{LOD_2(\theta_2)} + (1-\alpha_1-\alpha_2)] - \log_{10}[\alpha_1 10^{G} + \alpha_2 + (1-\alpha_1-\alpha_2)]$$

to the total likelihood, and a family in which mutation testing has not been performed contributes as described previously (see eq. [A2]).

Confidence intervals for the parameters α_1, α_2, $(1-\alpha_1-\alpha_2)$, and δ were computed by comparing the difference in log$_{10}$ likelihoods for different values of α_1, α_2, $(1-\alpha_1-\alpha_2)$, and δ, respectively, and maximizing over the other parameters, to a χ^2 distribution on 1 df. To compute the upper and lower confidence limits on the proportion of families with disease attributable to a gene (e.g., BRCA1), we used the upper and lower confidence limits, respectively, on α_1, with the maximum-likelihood estimate for α_2, given α_1, and with computed posterior probabilities of linkage to each gene, as described above.

Appendix C

Estimation of the Penetrance of BRCA2

Computations were performed with a modified version of the ILINK program (Lathrop et al. 1984); D13S267 typings were used, since the computer time that would be required for maximization over multipoint data was prohibitive. Incidence rates in noncarriers were fixed at the population rates for England and Wales. Rates in carriers were modeled under the assumption of a separate parameter for the ratio of the incidences in gene carriers versus the incidences for carriers in the CASH model, for each of five age groups (20–29 years, 30-39 years, 40–49 years, 50–59 years, and 60–69 years). Follow-up of all females was censored at age 70 years. Men were included in the liability class for the 20–29-years-old age group. Male breast cancer cases were therefore assumed to be likely to be gene carriers, regardless of age at diagnosis, and no attempt was made to estimate the penetrance for male breast cancer. The overall penetrance, defined as risk of breast or ovarian cancer, was modeled by defining age at onset as the age at diagnosis of the first cancer (either breast or ovarian). Estimates of breast and ovarian cancer incidence were then obtained by dividing the overall incidence rate in each age group by the observed age-specific proportions of breast and ovarian cancer.

The penetrance of BRCA2, as based on families with six or more breast cancers, was estimated by maximization of the LOD score

$$LOD(\gamma) = \Sigma \log_{10}[\gamma 10^{LOD_1(\theta_1)} + (1-\gamma)10^{LOD_2(\theta_2)-F}] - \Sigma \log_{10}[\gamma + (1-\gamma)10^{F}] ,$$

where γ is the proportion of high-risk mutations that are BRCA1 mutations. The proportion of high-risk mutations that are BRCA2 mutations is then forced to be $(1-\gamma)$. $LOD_1(\theta_1)$ is the LOD score at BRCA1, under the BRCA1 model (and is fixed), and $LOD_2(\theta_2)$ is the LOD score at BRCA2, under the model estimated for BRCA2 (which is maximized over different penetrance functions). $F = \log_{10}[L_1(D)/L_2(D)]$, where $L_i(D)$ is the likelihood of the disease phenotypes under the BRCA1

model (and is fixed) and $L_2(D)$ is the likelihood of the disease phenotypes under the BRCA2 model (and has to be recomputed at each iteration, as the model changes). (In practice we computed $F = \log_{10}[L_1(D,M/1/2)/L_2(D,M/1/2)]$ by using MLINK with D13S267 typings [when these were available] or, otherwise, a dummy marker with complete typing [for speed].)

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ELSEVIER

Radiotherapy and Oncology 47 (1998) 129–136

RADIOTHERAPY & ONCOLOGY
JOURNAL OF THE EUROPEAN SOCIETY FOR THERAPEUTIC RADIOLOGY AND ONCOLOGY

Response to radiation therapy and prognosis in breast cancer patients with BRCA1 and BRCA2 mutations[1]

David K. Gaffney[a,*], Richard M. Brohet[b], Cathryn M. Lewis[c], Joseph A. Holden[d], Saundra S. Buys[e], Susan L. Neuhausen[c], Linda Steele[c], Vilija Avizonis[f], John Robert Stewart[a], Lisa A. Cannon-Albright[c,e]

[a]*Department of Radiation Oncology, University of Utah, 50 N. Medical Dr., Salt Lake City, UT 84132, USA*
[b]*Department of Epidemiology, The Netherlands Cancer Institute, Amsterdam, The Netherlands*
[c]*Department of Medical Informatics, University of Utah, Salt Lake City, UT, USA*
[d]*Department of Pathology, University of Utah, Salt Lake City, UT, USA*
[e]*Department of Internal Medicine, University of Utah, Salt Lake City, UT, USA*
[f]*Radiation Center, LDS Hospital, Salt Lake City, UT, USA*

Received 11 September 1997; revised version received 11 March 1998; accepted 17 March 1998

Abstract

Background and purpose: The purpose of this study is to evaluate overall survival in BRCA1 or BRCA2 breast cancer patients, describe presenting stage, review histologic findings and evaluate response to radiotherapy.

Materials and methods: A retrospective study was performed evaluating breast cancer patients with known mutations of BRCA1 or BRCA2. Patients from 12 different pedigrees were cross-referenced with the Utah Cancer Registry (UCR), histologic findings were verified and radiotherapy records were reviewed for acute response to treatment. Actuarial survival calculations were performed and patients were matched for age, date of diagnosis and tumor size.

Results: Thirty breast cancer patients with BRCA1 mutations were found to have 34 breast cancers (four had bilateral metachronous lesions) and 20 breast cancer patients with BRCA2 mutations were found to have 22 breast cancers (two had bilateral metachronous disease). The median age at diagnosis was 49 years (range 21–77 years) and 42 years (range 23–83 years), respectively, for BRCA1 and BRCA2 patients. Unusual histologic types of breast cancers were represented with 7% (4/56) medullary and 5% (3/56) lobular carcinomas. Complete staging was possible for 63% (35/56) of cancers. Stages I, II, III and IV represented 26, 63, 6 and 6% of cancers, respectively. The most severe radiation reaction was moist desquamation which was self-limiting and developed in 29% (6/21) of irradiated patients. The mean follow-up was 9.8 and 7.5 years for BRCA1 and BRCA2 cancers, respectively. Kaplan–Meier survival analysis demonstrated 5-year survival values of 75% for BRCA1 patients, 73% for BRCA2 patients, 70% for matched controls and 69% for UCR controls. No statistically significant differences were evident between the groups at 5 or 10 years.

Conclusions: Despite their younger age at presentation, breast cancer patients harboring BRCA1 or BRCA2 mutations present at a similar stage, display a normal acute reaction to radiotherapy and have a similar prognosis when compared with sporadic breast cancer patients.

Keywords: Breast cancer; BRCA1; BRCA2; Pathology; Radiation; Prognosis

1. Introduction

The inherited basis of certain breast cancers has been confirmed with the description of three dominant genes, namely BRCA1, BRCA2 and p53. All display a loss of heterozygosity consistent with the role of a tumor suppressor gene. In one study of young women with breast cancer (age less than 30 years), 13% were found to have BRCA1 mutations [9]. For Jewish women less than 40 years old diagnosed with breast cancer, 21% were found to have the specific BRCA1 mutation, 185delAG [9]. It is possible that less than 10% of all breast cancer cases are due to these

* Corresponding author.

[1] This work was presented at the 21st meeting of the International Association for Breast Cancer Research, 3 July 1996, Paris, France.

0167-8140/98/$19.00
PII S0167-8140(98)00023-1

genes; however, important aspects of breast carcinogenesis may be revealed in these syndromes [10]. With the potential advent of widespread genetic testing, further description of these clinical syndromes is needed.

Through genetic linkage analysis BRCA1 was localized to 17q21 [13]. The BRCA1 gene contains 22 exons distributed over more than 100 kb of genomic DNA and encodes for a protein of 1863 amino acids [20]. Recently, both BRCA1 and BRCA2 have been associated with the Rad51 protein, which is involved in the integrity of the genome [27,29]. Reduced expression has also been identified in transformation to a neoplastic state, which is consistent with its role as a tumor suppressor gene [34]. Many mutations have been described with the majority resulting in a truncated protein [30]. The precise frequency of the mutated gene is unknown; however, it has been estimated to be approximately one in 800 in the normal population [24]. BRCA1 and BRCA2 mutations are identified in approximately 12 and 3% of young women with breast cancer, respectively [9,16].

BRCA2 has been identified on chromosome 13q12–13 and likely accounts for a large proportion of non-BRCA1 familial breast cancer [33,37]. BRCA2 has been implicated in male breast cancer and it displays the characteristic loss of heterozygosity (LOH) consistent with a tumor suppressor gene [35]. Additionally, LOH at 13q12–13 has been seen in a variety of different tumors from BRCA2 carriers suggesting a possible role in tumorigenesis [12].

Younger women with breast cancer have more aggressive lesions with an increased risk of relapse and death [14,21].

Table 1

Clinical parameters

	BRCA1	BRCA2
Age (years) (mean ± SD)	49 ± 14	46 ± 14
Tumor size (mm) (mean ± SD)	22 ± 13	31 ± 18
No. of positive lymph nodes (mean ± SD)	1.5 ± 3.0	2.6 ± 4.3
Follow-up (years) (mean ± SD)	9.8 ± 8.2	7.5 ± 5.3
No. of patients	30	20
No. of breast cancers	34	22
No. of kindreds	7	5
Lymph node (%)		
Negative	15 (44)	10 (45)
Positive	17 (50)	12 (55)
Unknown	2 (6)	
Stage (%)		
I	6 (18)	3 (14)
II	12 (35)	10 (45)
III	1 (3)	1 (4)
IV	1 (3)	1 (4)
Unknown	14 (41)	7 (32)
Grade (%)		
I	2 (6)	1 (4)
II	4 (12)	4 (18)
III	11 (32)	4 (18)
Unknown	17 (50)	13 (59)
Radiation Tx (%)	11 (32)	10 (45)
2nd Malignancy (%)	11 (37)	5 (25)

Tumor aggressiveness is manifest by an elevated S-phase fraction, abnormal p53 expression and higher histologic grade [1,18,19,22]. For BRCA1-related breast cancer, Eisinger et al. [8] noted that histopathologic grade appeared to segregate as a genetic trait, thus establishing a genotype–phenotype correlation.

On the other hand, familial breast cancer has been described to have a more favorable survival outcome than sporadic breast cancer [2,25]. In the study by Marcus et al. [18], BRCA1-related breast cancer patients had a higher proliferation rate, greater disease-free survival and similar overall survival compared with sporadic controls. Thus, an interesting dichotomy appears to exist for patients with BRCA1-related breast cancer where pathologic analysis yields a more aggressive grade while survival is improved or comparable to sporadic cases.

The objectives of this report are to analyze overall survival in patients with defined BRCA1 and BRCA2 mutations, review histologic findings and describe the response to radiation therapy.

2. Methods

2.1. Ascertainment of patients

This study used patients who had previously been used in linkage analysis to localize BRCA1 or BRCA2 [20,30,33]. We included all patients with breast cancer found within the state of Utah who were BRCA1 or BRCA2 mutation carriers. Patients were cross-referenced with the Utah Cancer Registry (UCR). The UCR has complete statewide cancer incidence data since 1966 and has over 148 000 cases. Thirty BRCA1 patients were identified from seven kindreds and 20 BRCA2 patients were identified from five kindreds. All breast cancer cases from within the above pedigrees were utilized and cases were not limited to living patients. All patients with BRCA1 or BRCA2 mutations were identified because of strong family histories of breast and/or ovarian cancer suggesting a highly penetrant autosomal dominant gene and consequently do not represent a cross-section of the population. Informed consent of patients or next of kin under the guidelines of the Institutional Review Board at the University of Utah was obtained for all patients for whom radiotherapy charts, pathology reports and samples were obtained.

2.2. BRCA1 and BRCA2 status

Germ-line mutations were identified for 6/7 BRCA1 kindreds and 3/5 BRCA2 kindreds (Tables 1 and 2). Ten patients from the other three kindreds were included because linkage evidence supported a high probability of disease due to the respective susceptibility. Identification of BRCA1 and BRCA2 mutations was by full sequencing of germ-line DNA. Each kindred had a unique mutation

Table 2
Mutations and crude death rates by kindred

Kindred	Mutation[a]	Confirmation (number)[b]	No. dead/total	Follow-up (months) (mean)
BRCA1				
1001	SP, ins 59	1M	0/1	23
1901	FS, 188 del II	1M	1/1	37
1910	FS, 5382 ins C	1M	0/1	324
2082	NS, Gln ter 1313	3M, 8I, 1L	9/12	81
2301	SP, ins 59	3M, 4I	4/7	147
2305	FS, 2982 del 5	2M, 2I	2/4	85
2331	Lod score 0.36	4L	0/4	142
BRCA2				
1018	FS, 982 del 4	2M, 3I	4/5	72
2044	FS, 4766 del 4	3M, 1I	1/4	95
2327	Lod score 1.92	5L	3/5	116
2388	Lod score 0.92	2L	0/2	44
107	FS, 277 del AC	3M, 1L	1/4	79

[a]SP, splice site mutation; FS, frame shift; ins, insertion; del, deletion.
[b]M refers to direct sequence confirmation of the specific mutation. I indicates an inferred mutation because of mutational confirmation in a child. L refers to linkage.

with the exception of kindreds 1001 and 2301. The description of the mutations has been published previously and is presented briefly in Table 2 [30,33].

2.3. Pathologic analysis

Pathologic samples were obtained from various facilities within the state of Utah for 29/56 lesions (six of the 50 patients had bilateral disease). Cases were accrued from 13 different hospitals within the state of Utah. Reasons for not obtaining pathology included inability to obtain consent for the release of slides (one patient), or unavailability of slides and tissue blocks (26 cases). The mean year for diagnosis of all cases was 1979 and the mean year of diagnosis for the patients for whom pathology was available was 1984 ($P = 0.07$). The samples were scored in a blinded fashion for histologic grading according to the Contesso recommendations of the Scarff, Bloom and Richardson system with three parameters, i.e. tubular differentiation (throughout, 1; occasional, 2; not seen, 3), nuclear pleomorphism (uniform and regular size, 1; moderate pleomorphism, 2; very pleomorphic with giant nuclei, 3) and mitotic index (less than one mitosis, 1; two mitoses, 2; more than three mitoses, 3). The final grade is determined by summing the three scores as follows: grade 1, 3–5; grade 2, 6–7; grade 3, 8–9 [7].

2.4. Clinical follow-up

Staging and follow-up was obtained from the UCR. Complete American Joint Committee on Cancer staging was present for 63% of the tumors. The remainder of the patients could not be fully staged because many of the earlier pathology reports and/or medical records did not include the primary tumor size. Patients were diagnosed with breast cancer from 1957 to 1994. Follow-up information was obtained on all patients through the UCR and no patients were lost to follow-up. This data base is updated annually.

Radiation therapy was utilized in 26/50 (52%) patients and 21/26 (81%) of these charts were reviewed. Reasons for not reviewing treatment records included inability to obtain medical consent for release of records (one patient) and inability to locate the chart (four patients). Patients were treated in five different facilities in Utah. Mastectomy was the surgical procedure for 49/56 (88%) tumors. Photon energies ranged from Cobalt to 6 MV and electron energies ranged from 6 to 11 MeV.

2.5. Statistical analysis

Statistical analysis of survival data and associated parameters was performed using χ^2 analysis and Kaplan–Meier survival curves. Data for the control group were obtained from the UCR. All cases in this study, as well as patients with multiple breast tumors, were removed from the control pool so that they were not scored twice. For each case, controls were selected from the UCR matching for age (±4 years), date of diagnosis (±4 years) and tumor size (±10 mm). Each case was matched with multiple controls (median 117, range 11–937). Controls could not be matched for axillary node status or stage because changes within the UCR coding system prevented data acquisition in this fashion. Tumor size, however, is an independent prognostic factor for the frequency of axillary node positivity, distant metastasis and survival [6]. If case patients did not have a designation of a primary tumor size (13/30 BRCA1 cases and 7/20 BRCA2 cases), they were matched for age and date of diagnosis only. Cases with metachronous bilateral breast cancer were matched on their initial diagnosis of breast cancer. Patients dying from causes other than breast cancer were censored at the time of death. The cause of death was obtained from death certificates and in some cases from a review of the medical records. Statistics were calculated using SAS (SAS Institute, Cary, NC).

3. Results

3.1. Clinical parameters

The median and range for the ages of onset were 49.5 and 21–77 years for BRCA1-related breast cancer and 42 and 23–83 years for BRCA2-related breast cancer, respectively (Table 1). For the UCR control group ($n = 18\,278$) the mean age at presentation was 60 years ($P = 0.0001$ versus the combined BRCA1 and BRCA2 group). The majority of patients had stage I or II disease and 52% (29/56) had positive axillary lymph nodes. In our case population of BRCA1- and BRCA2-related breast cancer T sizes 1 (tumor size 0–20 mm), 2 (21–50 mm) and 3 (>50 mm)

Table 3
Histologic parameters

	Group		
	BRCA1	BRCA2	Control[a]
Ductal	19 (56)	14 (64)	4621 (55)
Adeno NOS	4 (12)	4 (18)	1601 (19)
Medullary	3 (9)	1 (4)	221 (3)
Carcinoma NOS	3 (9)	1 (4)	509 (6)
Lobular	2 (6)	1 (4)	285 (3)
Signet ring adeno	–	1 (4)	6 (<1)
Intraductal	2 (6)	–	277 (3)
Malignant NOS	1 (3)	–	155 (2)
Other			732 (9)

Numbers in parentheses are percentages.
[a]The control group represents the BRCA1 and BRCA2 group matched for age, date of diagnosis and tumor size.

represented 39, 51 and 9% of cases, respectively. The T sizes matched closely with a large retrospective study of over 24 000 sporadic breast cancer cases where the corresponding percentages for T sizes 1, 2 and 3 were 34, 55 and 11%, respectively [6]. The incidences of axillary nodal positivity in our case population for T sizes 1, 2 and 3 were 31, 43 and 67%, respectively, which corresponds to an incidence of 31, 50 and 70%, respectively, in sporadic breast cancer cases [6].

Contralateral breast cancer developed metachronously in 13% (4/30) and 10% (2/20) of patients with BRCA1- and BRCA2-related breast cancer, respectively. The time interval ranged from 0.6 to 22.8 years for the BRCA1 cases and from 2.9 to 3.3 years for the two BRCA2 cases.

The majority of cancers were invasive ductal carcinomas. The incidences of medullary carcinomas for the BRCA1 group, BRCA2 group and matched control group were 3/34 (9%), 1/22 (4%) and 221/8409 (2.6%), respectively (BRCA1 versus matched control group, $P = 0.060$, Fisher's exact test, Table 3). The incidences of invasive lobular carcinomas for the BRCA1 group, BRCA2 group and matched control group were 2/34 (6%), 1/22 (4%) and 285/8409 (3.4%), respectively.

Of 56 BRCA1- or BRCA2-related breast cancers, 26 (46%) were evaluable for histologic grading (Table 1). Grade 3 tumors were found in 65 and 44% of BRCA1- and BRCA2-related breast cancers, respectively. BRCA1 patients with a mitotic index of 1 ($n = 6$) were significantly older than patients with mitotic scores of 2 and 3 ($n = 11$, $P = 0.008$) with mean ages of 55 versus 41 years, a finding similar to that of Eisinger et al. [8]. When BRCA2-related patients were included, there was no significant difference ($P = 0.425$). Grade did not correlate with survival. The overall survival for BRCA1 patients with grade 1, 2 and 3 cancers was 50, 50 and 45%, respectively.

3.2. Radiation response

Twenty-one courses of radiation therapy from 1960 to 1994 were reviewed. Breast conservation therapy was performed in seven cases and postmastectomy radiation was performed in 14 cases. The dose was 52.3 ± 5.5 Gy (mean ± standard deviation). Radiation treatment was delivered in 25 ± 5 fractions and the number of elapsed days was 37 ± 7. The median dose per fraction was 200 cGy and the range was 180–323 cGy. A variety of techniques was employed (Table 4). Responses to radiation were obtained retrospectively from treatment notes, treatment summaries and follow-up visits from the radiation therapy charts. Typical responses to chest wall and breast irradiation were observed (Table 5). Moist desquamation developed in 3/11 (27%) BRCA1 patients and in 3/10 (30%) BRCA2 patients. Bolus was utilized in 6/14 (43%) postmastectomy patients. All patients who developed moist desquamation did so toward the completion of radiation treatment. The incidence of moist desquamation in BRCA1 and BRCA2 patients compared favorably with values reported in the literature and at our institution (Table 6) [3,11,15,26,36]. Due to concerns of significant acute reactions, 2/21 patients had a treatment break and one patient had treatment curtailed three fractions early because of brisk erythema. One patient developed an idiopathic rash that healed spontaneously and one patient who had a mastectomy and was irradiated with tangents to the chest wall and an anterior port to the supraclavicular fossa developed moderate arm edema. Late sequelae, such as radiation pneumonitis, pericarditis, rib fractures, skin necrosis or second malignancies in-field, were not reported.

Breast conservation therapy (BCT, limited surgery and radiation to the intact breast) was performed in three

Table 4
Radiation therapy treatment technique

	n (%)	Target area
Tangents (T)	5 (25)	CW
Hockey stick (H)	5 (25)	SC, IMN
Electrons (E)	2 (10)	CW
T and H	4 (20)	CW, SC, IMN
T and SC	2 (10)	CW, SC
E and H	2 (10)	CW, SC, IMN

Mastectomy was the surgical procedure for 49/56 (88%) tumors.
H refers to ipsilateral supraclavicular (SC) and internal mammary nodal fields (IMN). E were treated enface. CW refers to chest wall. Photon energies ranged from Cobalt to 6 MV and E energies ranged from 6 to 11 MeV.

Table 5
Acute radiation reactions

	n (%)
Moderate erythema	8 (38)
Brisk erythema	2 (10)
Dry desquamation	1 (5)
Moist desquamation	6 (29)
Treatment break	2 (10)

Table 6
Radiotherapy-induced moist desquamation

Reference	Dose (Gy)	Technique	*n*	% MD	Comments
Turesson and Thames [36][a]	24–55	Enface	764	19	Multiple fraction schemes
	44–52	Enface	188	38	Conventional fraction
Bentzen and Overgaard [3][b]	37–51	Electrons	229	16	Only patients with >10% MD
Hiraoka et al. [15]	50–60	Tangents	194	11	No wedge (Co^{60} used)
	50–60	Tangents	145	7	Wedge (Co^{60} used)
Sause et al. [26][c]	45	Tangents	22	0	4 MV photons
	40	Enface	33	12	7 MeV electrons, 4 Gy × 10
Gaffney et al. [11][c]	24–70	Electron arc	150	27	Rotational therapy
This study	45–64	Variety	21	29	BRCA1 and BRCA2

n refers to the number of patients in the study and MD indicates moist desquamation.
[a]Conventional fraction refers to ≤2 Gy per day. Patients were treated either with 12 or 13 MV electrons or 200 kV photons.
[b]Only patients with >10% of the field with MD were included.
[c]Patients were treated postmastectomy.

BRCA1 patients (one patient had treatment for bilateral cancers) and in three BRCA2 patients with a mean follow-up time of 46 months. The patient with bilateral metachronous disease experienced a local failure at 24 months which was treated by bilateral mastectomy and focal re-irradiation due to concern of a close surgical margin. One patient had stage IV disease and died of metastatic disease. Thus, six of seven treated breasts were locally controlled.

3.3. Survival

Kaplan–Meier survival analysis demonstrated 5-year actuarial survival values of 75% for BRCA1 patients, 73% for BRCA2 patients, 69% for UCR controls and 70% for controls matched for age, date of diagnosis and tumor size (Figs. 1 and 2 and Table 7). There was no statistically significant difference in survival by the log-rank test between these groups. There were four non-cancer causes of death, i.e. Parkinson's disease (kindred 2301) and heart disease (two patients in kindred 2082 and one patient in kindred 2327). There were 17 deaths from breast cancer, two from ovarian cancer, one from uterine cancer and one from lymphoma.

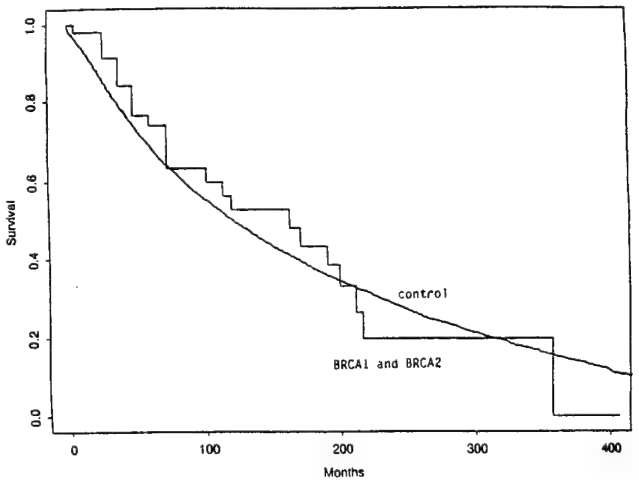

Fig. 1. Kaplan–Meier overall survival curves of BRCA1- and BRCA2-related breast cancer patients (solid line) versus Utah Cancer Registry controls (dashed line).

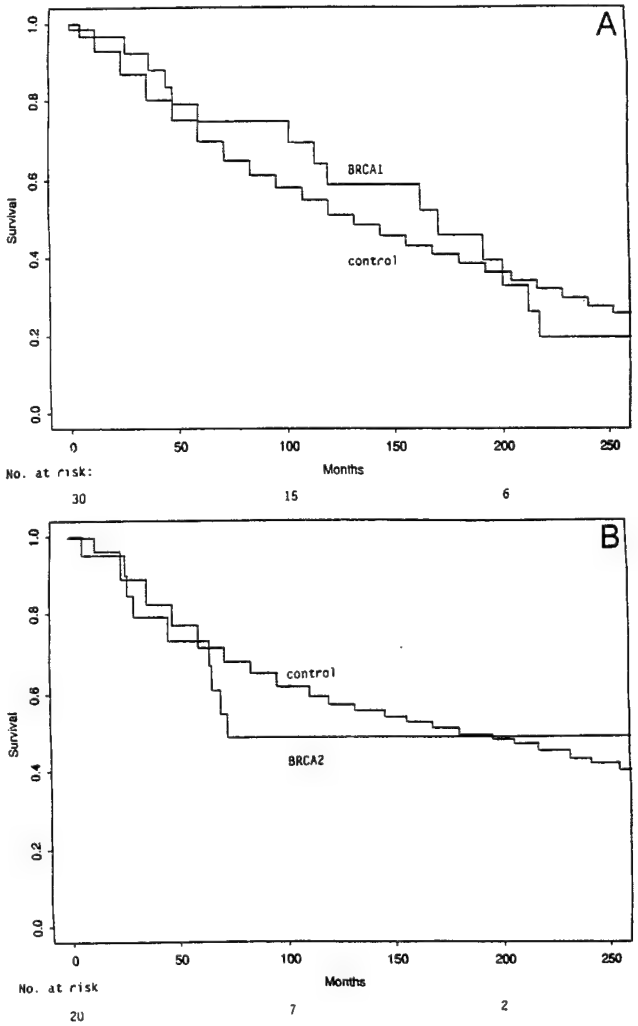

Fig. 2. Kaplan–Meier overall survival curves of BRCA1-related breast cancer patients (solid line (A)) and BRCA2-related breast cancer patients (solid line (B)) versus controls (dashed line). The controls were breast cancer patients obtained from the Utah Cancer Registry and were matched for date of birth (± 4 years), date of diagnosis (± 4 years) and tumor size (± 10 mm).

Table 7
Five-year survival in hereditary breast cancer

Reference	Year	Five-year overall survival (%)		Comments
		Cases	Controls	
Albano et al. [2]	1980	106 (67)	24136 (45)	Vertical transmission
Porter et al. [25]	1994	35 (83)	910 (61)	17q linked
		24 (59)		Not 17q linked
Marcus et al. [18][a]	1996	72 (67)	181 (59)	17q linked
		66 (63)		Some 13q linked
Sigurdsson et al. [31][b]	1996	42 (40)	115 (55)	BRCA2 linked
This study	1996	30 (75)	17396 (69)	BRCA1 87% genotyped
		20 (73)		BRCA2 65% genotyped

[a]The survival calculations represent crude values, whereas the others report actuarial values.
[b]The survival values represent 10-year disease-free survival.

The crude death rate per kindred or allelic heterogeneity revealed a large variance which is likely due to small sample sizes. Twelve different kindreds were represented with the number of patients ranging from one to 12 with a median of four per kindred. Cancer was the cause of death in 43 and 40% of patients with BRCA1 and BRCA2 mutations, respectively.

4. Discussion

Patients with hereditary breast cancer present frequently at an earlier age. Interestingly, our study patients more closely approximated population-based studies than previous reports [8,18,25]; nonetheless, they presented on average more than a decade younger compared with a population-based control group. These study patients were identified because of strong family histories of breast and/or ovarian cancer suggesting a highly penetrant autosomal dominant gene and consequently do not represent a cross-section of the population. An additional caution in the interpretation of these results is that 21 patients were treated with chemotherapy and 11 patients were treated with hormonal agents. Patients were diagnosed with breast cancer over a 37-year span and there was no consistent policy in the administration of chemotherapy. Due to the retrospective nature of case ascertainment multiple scenarios for bias exist and ultimately prognosis and the side-effects of treatment of patients with hereditary breast cancer should be obtained from prospective data. Potential advantages of studying these extended families with segregating mutations include multiple cases with the same mutations, similarity of lifestyle risk factors and a relative common environmental exposure, since all cases were accrued from within the state of Utah.

Over 125 mutations have been identified in the BRCA1 gene and mutations have been described over the entire gene and involve coding and non-coding sequences. Most mutations identified to date result in a truncated form of the protein and a hypothesized cause of variable risk due to different mutations has been the length of the protein product [30].

Localized disease (lymph node-negative) was present in 45% (25/56; in two cases the lymph node status was unknown) of cases in our study and in 62% (45/72) of cases reported by Marcus et al. [18]. In the USA between 1986 and 1991, 58% of breast cancers were localized disease [23]. Whether the stage at presentation is altered by surveillance strategies for women at very high risk for breast cancer is currently unknown. Both infrequent and excessive surveillance behaviors have been reported in women at high risk [17]. This is a crucial issue since surveillance by mammography has been demonstrated to reduce breast cancer mortality in women greater than 50 years old and the optimal surveillance strategy for women at high risk has not been defined.

Additionally, both an increase in 5-year survival and a higher proportion of early breast cancer cases have been noted over the past decades [21]. Thus, it is important to control for these variables and this study demonstrated no change in overall survival when the above variables were controlled. Due to the small sample size in our study, caution is warranted in making definitive conclusions.

The grade of BRCA1-related breast cancers has been shown to be consistently elevated with grade 3 comprising 61.3–81.5% of cases versus 22–27% of hospital-based comparison series [8,18]. In this report, 65% of tumors from BRCA1 patients were high grade. Although a higher proportion of grade 3 tumors has been reported for BRCA1-related breast cancers [8,18], survival has been reported as comparable or better [18,25]. Additionally, hereditary breast cancer presents at a slightly younger age [1,18,25] and a young age has been demonstrated to be an independent adverse risk factor [1,14,21,22]. Thus, grade 3 in BRCA1-related breast cancer may have a different implication for prognosis than in other breast cancer cases [4].

Several reports have suggested that the prognosis for BRCA2-related breast cancer may be more guarded [18, 25,31]. In our study BRCA2 patients had a lower 10-year survival (59 versus 49%, BRCA1 versus BRCA2, respectively, $P = 0.279$, χ^2), which was not significant.

Patients with BRCA1- or BRCA2-related breast cancer do not have an exacerbated acute response to radiation (Table 6); again, however, due to the limited sample size our conclusions are preliminary. Although the fractionation schemes and doses differed over the years, a mean of 37 days to complete treatment suggest that patients did not have excess acute toxicity that delayed the completion of treatment. Only 12% (7/56) of cases were treated with BCT and one patient experienced a local failure. The mean year for diagnosis was 1979 and consequently many patients were treated before BCT was demonstrated to be a safe

alternative to mastectomy. The safety of BCT in BRCA1- and BRCA2-related breast cancer treatment is supported by extended disease-free survival compared with controls as reported by Marcus et al. [18] and overall survival is not worse [2,18,25]. Hence, therapeutic recommendations that are more limited are not congruous with the known data. Admittedly, there is a paucity of data on the subject. Young women display a higher but not statistically significant local failure rate [32]. Consequently, a young age is not a relative contraindication to BCT. Knowledge of the response to adjuvant therapies in breast cancer is a critical issue, specifically in hereditary breast cancer.

Contralateral breast cancer is the most frequent second cancer in breast cancer patients and patients with hereditary breast cancer are at increased risk for development of contralateral breast cancer. It is unclear whether breast irradiation leads to a higher relative risk for the development of contralateral breast cancer [28]. Boice et al. [5] reported a relative risk of 1.59 for development of contralateral breast cancer in women less than 45 years old. The average radiation dose to the contralateral breast was 2.8 Gy and the associated risk was dose-dependent [5]. Other studies have demonstrated no effect of age on the risk of contralateral breast cancer development. A minimum latency of 10 years likely exists for the development of contralateral breast cancer and consequently many of the data from randomized trials may be premature for analysis for this effect. Due to the low incidence of breast cancer in young women, particularly with hereditary breast cancer, and the low expected relative risk, limited statistical power may exist to address this issue.

In conclusion, breast cancer patients harboring BRCA1 or BRCA2 mutations present at a similar stage, display a normal reaction to radiotherapy and have a similar prognosis when matched for age, date of diagnosis and tumor size when compared with sporadic breast cancer patients. Two subgroups of BRCA1-related breast cancers may exist, i.e. a smaller subgroup with late onset and a low mitotic index and a larger portion of cases manifest by early onset and a high proliferation rate.

Acknowledgements

This study was supported in part by grants CA55914, Army DAMD 17-94-J4260 and CA96446 and contract NO1-CN05222.

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Pathobiologic Characteristics of Hereditary Breast Cancer

BEVERLY J. LYNCH, MD, JOSEPH A. HOLDEN, MD, PHD, SAUNDRA S. BUYS, MD, SUSAN L. NEUHAUSEN, PHD, AND DAVID K. GAFFNEY, MD, PHD

Patients with hereditary breast cancer (HBC) present at a young age with breast cancers that show adverse pathological characteristics such as high nuclear grade, negative hormone receptor status, and high proliferation indices. Surprisingly, the clinical course has been reported to be comparable or improved compared with patients with nonhereditary breast cancer (non-HBC). To determine whether there are any molecular markers that might help explain this paradox between pathologically aggressive neoplasms in patients with HBC and the lack of extreme clinically aggressive disease, we studied several molecular parameters in a group of 34 breast cancer patients with mutations in either the BRCA1 or BRCA2 tumor suppressor genes and compared them with a group of 20 breast cancer patients with non-HBC. In general, patients with HBC had tumors that were of higher nuclear grade, contained a higher population of proliferating cells, showed increased expression of DNA topoisomerase II-alpha (topo II-alpha), lacked hormone receptors, and were more likely to show immunopositivity for the *p53* tumor suppressor gene. Additionally, tumors from patients with HBC showed a decreased angiogenesis compared with controls. The decreased angiogenesis and the elevated expression of topo II-alpha (an anticancer drug target) may, in part, explain the lack of correlation between clinical course and histological characteristics in patients with HBC. HUM PATHOL 29:1140-1144.

Key words: hereditary breast cancer, BRCA1, BRCA2, immunohistochemical staining, DNA topo II-alpha.

Abbreviation: HBC, hereditary breast cancer.

Breast cancer is the leading malignancy in women and the second most common cause of cancer-related deaths in the United States.[1] Observations of a family history of breast cancer with an early age of onset spurred research into the investigation of specific genes that may be responsible for the development of this disease. As a result, BRCA1 and BRCA2, the two genes that appear to confer susceptibility to the development of breast carcinoma, have been isolated and characterized. The BRCA1 gene has been mapped to chromosome 17q12-21, and the BRCA2 gene has been mapped to chromosome 13q12-13.[2,3] Together, these two genes probably account for the majority of hereditary breast cancer (HBC), or 5% to 10% of all breast cancers.[4,5]

The clinical aspects and the pathological characteristics of the neoplasms in patients with HBC have not been widely studied. From the limited data available, it appears that patients with HBC may have a better than or similar prognosis to patients with sporadic tumors.[6-8] This result is surprising because several studies have indicated that breast cancers arising in patients with HBC have pathological characteristics such as high nuclear grade, high proliferation indices, absent hormone receptor status, and increased p53 immunopositivity; features that are usually associated with more aggressive disease.[7,9-13]

In an effort to understand more fully this apparent paradox between relatively favorable clinical course and poor pathological indicators, we evaluated the pathological characteristics of breast carcinoma in 21 patients with known BRCA1 mutations and in 13 patients with known BRCA2 mutations and compared them with the pathological characteristics observed in breast carcinoma from 20 patients with non-HBC. The patients selected for comparison were consecutive cases obtained from a single institution and were not matched to the case groups. Several new histological parameters that may have important prognostic implications in breast cancer, and have not been previously studied in this group of tumors, such as DNA topoisomerase II-alpha and tumor microvessel density, have been evaluated.[14,15]

MATERIALS AND METHODS

Patient Characteristics

Breast cancer tissue was available from 21 patients with BRCA1 mutations (one patient had metachronous, bilateral breast cancers, and consequently, there were a total of 22 cases), and from 13 patients with BRCA2 mutations, representing nine BRCA1 families and six BRCA2 families (Table 1). Each identified mutation was unique, with the exception of kindreds 1001 and 2301.[16,17] Germline mutations were identified by full genomic sequencing for 20 of 21 (95%) of BRCA1 patients and 10 of 13 (77%) of BRCA2 patients. The other cases were included based on a high lod score and shared haplotype among breast cancer cases (Table 1). A group of 20 sporadic cases of breast cancer were retrieved from the surgical pathology files at the University of Utah and were not matched to the BRCA1 or BRCA2 cases. The genotype status of the patients was blinded to the reviewing pathologist. All

From the Departments of Pathology, Internal Medicine, Medical Informatics, and Radiation Oncology, University of Utah, Salt Lake City, UT. Accepted for publication March 30, 1998.

Supported by DOD grants DAMD-17-94-J-4260 and DAMD 17-96-I-6266 and NIH grant R01-55914 (S.L.N.) and a Huntsman Cancer Institute grant (D.K.G.). It was also supported by the Utah Cancer Registry, which is funded by Contract #N01-CN-6700 from the National Cancer Institute with additional support from the Utah Department of Health and the University of Utah.

Presented at the 39th Annual Meeting of the American Society for Therapeutic Radiology and Oncology, Orlando, FL, October 20, 1997.

Address correspondence and reprint requests to David K. Gaffney, MD, PhD, University of Utah, 50 N Medical Dr, Salt Lake City, UT 84132.

0046-8177/98/2910-0019$8.00/0

TABLE 1. Mutations

Kindred	No. of Patients	Mutation
BRCA1		
1001	1	SP-FS, IVS 5 (−11, T > G, 59 bp ins)
1901	1	FS, 188 del 11
2035	4	Del, 14 Kb
2082	5	NS, Gln 1313 ter
2099	2	MS, Met 1775 Arg
2301	3	SP-FS, IVS 5 (−11, T > G, 59 bp ins)
2305	3	FS, 2982 del 5
2331	1	Linked
2373	1	FS, 3875 del 4
BRCA2		
107	3	FS, 277 del AC
1018	2	FS, 982 del 4
2044	3	FS, 4766 del 4
2327	2	lod score 1.92
2367	2	SP, IVS 2 (+1, G > A)
2388	1	lod score 0.92

Abbreviations: SP, splice site; FS, frame shift; NS, nonsense; MS, missense; IVS, intervening sequence; del, deletion; ins, insertion.

slides were reviewed to confirm the diagnosis and given a modified Bloom-Richardson score.[18] The use of human tissue for this work was approved by the Institutional Review Board at the University of Utah.

Chemicals and Antibodies

The source of the chemicals and antibodies used were as described.[14] In addition, antibodies against the von Willebrand factor and p53 (clone DO-7) were from DAKO (Carpinteria, CA).

Immunohistochemical Staining and Interpretation

Immunohistochemical staining of histological sections prepared from human breast cancers was performed as described in detail elsewhere.[14] Briefly, slides were deparaffinized and heated (except for HER2/neu, which does not require the heating step) in 10 mmol/L sodium citrate (pH 6.0) for 30 minutes in a microwave oven. After cooling, immunohistochemical staining was performed with the use of a Ventana 320 automated immunohistochemical stainer in accord with the manufacturer's instructions. Detection was with a secondary mouse anti-immunoglobulin linked to biotin followed by incubation with streptavidin linked to horseradish peroxidase. Color development was accomplished with diaminobenzidine as the chromogen.

The dilutions of the antibodies used in immunohistochemical staining were as follows: topo II-alpha, 1:500; MIB1, 1:40; estrogen and progesterone receptors, 1:60; Her2/neu (c-erb-2): 1:800; Factor VIII: 1:1600; p53, 1:80.

Topo II-alpha and MIB1 were expressed as the topo II-alpha or MIB1 index, respectively. This was performed as described and represents the percent of positive staining cells.[14] Evaluation of p53 expression was performed in a similar fashion. At least 500 tumor cells were counted, and the number of positive p53 staining cells was determined. Evaluation of p53 immunostaining has not yet been standardized. Authors have used as little as 1% to greater than 20% cell positivity as a positive interpretation, suggesting gene mutation and accumulation of mutant protein.[12,19-21] Independent research evaluating neuroendocrine lung tumors and breast carcinomas found greater than 20% positivity to be significant both for missense mutations as well as patient prognosis.[20,21] Therefore, in this study, neoplasms that contained greater than 20% nuclear immunostaining were considered positive, and tumors that contained 20% or less immunostaining were considered negative. Overexpression of Her2/neu was observed by noting any distinct membrane staining of the tumor cells as described.[14] Hormone receptor staining was interpreted as positive if nuclear staining was observed in greater than 20% of the cells, and negative when 20% or fewer of the cells showed positive staining. Microvessel density was determined as described.[22] After staining with factor VIII, the slide was evaluated to determine the area with the highest intensity of staining. The number of vessels were counted in four 20× fields. The lowest count was discarded, and the remaining three counts averaged and expressed as the number of vessels divided by the size of the microscopic field.

Statistics

For continuous, numerical values, a *t*-test was used to compare groups. Otherwise, chi-square or Wilcoxon rank-sum test were applied.[23] Statistics were performed with the use of Statworks (Abacus Concepts, Inc., Berkeley, CA), Macintosh computer program.

RESULTS

Clinicopathologic Features of Patients With HBC and Non-HBC

Breast cancer develops at an earlier age in HBC than in non-HBC. The median age of onset was 42.4 years in BRCA1 patients ($P < .001$, versus sporadic controls), 48.4 years in BRCA2 patients ($P = .04$, versus sporadic controls), and 60.6 years in sporadic cases (Table 2). Patients with HBC have tumors of higher grade ($P < .001$ and $P = .009$ for BRCA1 cases and BRCA2 cases, respectively). The mitotic score was increased in the BRCA1 group versus the control group ($P = .003$). The BRCA2 group had more tumors with a

TABLE 2. Clinicopathologic Features of Hereditary and Nonhereditary Breast Cancer

	BRCA1	BRCA2	Non-HBC
Age			
(mean)	42.4†	48.8*	60.6
(range)	(21-63)	(34-78)	(33-87)
Tumor grade			
1	1 (4%)	4 (31%)	7 (35%)
2	7 (32%)	6 (40%)	10 (50%)
3	14 (64%)†	3 (23%)†	3 (15%)
Mitotic score			
1	5 (22%)	6 (45%)	17 (85%)
2	3 (14%)	3 (23%)	0 (0%)
3	14 (69%)†	4 (31%)	3 (15%)
Estrogen receptor			
Positive	5 (23%)	5 (38%)	16 (80%)
Negative	17 (77%)†	8 (62%)	4 (20%)
Progesterone receptor			
Positive	5 (23%)	5 (38%)	16 (80%)
Negative	17 (17%)†	8 (62%)	4 (20%)

NOTE. *P* values represent differences compared with the non-HBC group.
*$P < .05$.
†$P < .005$.

mitotic score of 3, but the difference was not statistically significant ($P = .075$). Additionally, the BRCA1 group also displayed an increase in nuclear pleomorphism versus sporadic controls ($P = .043$, data not shown). Medullary features were identified in 2 of 22 (9%) BRCA1 cases and in zero BRCA2 cases. Lobular features were seen in 2 of 22 (9%) BRCA1 cases and 1 of 13 (8%) BRCA2 cases. Tubular differentiation was seen in 2 of 22 (9%) BRCA1 cases and 3 of 13 (23%) BRCA2 cases. For both BRCA1- and BRCA2-related breast cancer, there was a decrease in estrogen and progesterone receptor positivity versus sporadic controls, but only the BRCA1 population was statistically different (Table 2).

Proliferation Markers in HBC and Non-HBC

Because of the higher mitotic scores in tumors from patients with HBC, we postulated that these neoplasms would express higher levels of the proliferation markers, topo II-alpha, and MIB1, than would tumors from patients with non-HBC. The average topo II-alpha index of 53 and MIB1 index of 57 for BRCA1 tumors is significantly higher than the topo II-alpha index of 24 and MIB1 index of 29 for the sporadic tumors (both $P < .001$, Table 3). Tumors from patients with BRCA2 mutations fall between these two values with an average topo II-alpha index of 35 and an average MIB1 index of 40. As shown in Figure 1, topo II-alpha indices correlate well with MIB1 indices in all of the breast cancers groups studied (correlation coefficient, $R = .93$).

Her2/neu, p53, and Microvessel Density in HBC and Non-HBC

Expression of Her2/neu was a relatively rare event in all of the breast cancers studied and was not statistically different between patients with HBC and non-HBC. As shown in Table 3, only one tumor with a BRCA1 mutation, one tumor with a BRCA2 mutation, and three tumors in the control population expressed this oncogene. In contrast, tumors from patients with HBC showed an increased frequency of p53 immunopositivity. Using a cutoff point of 20% as shown in Table 3, 10% of sporadic tumors were p53 immunopositive, whereas 36% of BRCA1 patients and 38% of BRCA2 patients were p53 positive ($P = .04$ and $P = .05$, respectively; chi-square analysis). If p53 positivity was compared as a continuous variable, the BRCA1 and BRCA2 groups retained statistical significance ($P = .021$ and $P = .012$, respectively) compared with the non-HBC group. However, if a cutoff point of 10% was applied, the BRCA1 group remained statistically significant ($P \leq .05$), whereas the BRCA2 group did not. The microvessel density was less in tumors from HBC patients than in tumors from non-HBC patients. The average microvessel density score was 15.5 in BRCA1 patients ($P = .03$) and 14.6 in BRCA2 patients versus 22.7 seen in patients with non-HBC.

TABLE 3. Immunohistochemical Staining Characteristics of Hereditary and Nonhereditary Breast Cancer

	BRCA1	BRCA2	Non-HBC
Topo II alpha (mean ± SD, %)	53 ± 26†	35 ± 22	24 ± 19
Mib1 (mean ± SD, %)	57 ± 28†	40 ± 18	29 ± 19
Her2/neu expression			
Positive	1 (5%)	1 (8%)	3 (15%)
Negative	21 (95%)	12 (92%)	17 (85%)
p53 Immunopositivity			
Positive	8 (36%)*	5 (38%)*	2 (10%)
Negative	14 (64%)	8 (62%)	18 (90%)
Microvessel density Microvessels/mm^2 (mean ± SD)	15.6 ± 7.8*	14.6 ± 9.9	22.7 ± 11.8

NOTE. P values represent differences compared with the non-HBC group.
*$P \leq .05$.
†$P \leq .005$.

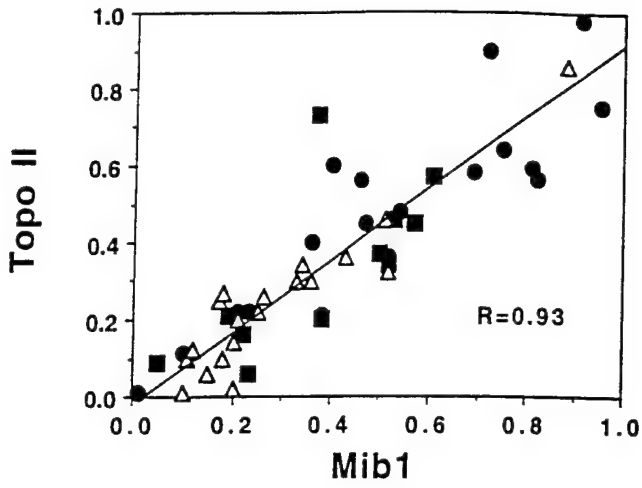

FIGURE 1. *Correlation of the topo II-alpha and MIB1 indices in HBC and non-HBC.* The topo II-alpha and MIB1 indices were determined as described in Materials and Methods. They have been divided by 100 and expressed as the fraction of positive staining tumor cells. The correlation coefficient between the topo II-alpha index and MIB1 index is 0.93 (● = BRCA1; ■ = BRCA2; △ = non-HBC).

DISCUSSION

In this work, we evaluated the pathological and clinical features of breast cancer arising in patients with HBC. Several points of caution are indicated in interpreting these results. Patients were accrued from families at high risk for HBC, and consequently do not represent a cross section of the population. The number of patients included is small; hence, the statistical power is limited. The control group was not matched for any prognostic factors such as stage, age, receptor status, or nodal status. Thus, multiple biases are possible. It was a series of sequential cases at a single hospital, and as such, it allows comparison with other series. Additionally, the use of "cutoff" values is not uniform in the literature. Both the BRCA1 and BRCA2 groups were significant compared with the non-HBC group with a cutoff point of 20%; however, the BRCA2 group lost significance with a cutoff point of 10%. The selection of a cutoff value for interpreting p53 positivity in breast carcino-

mas is critical to select differences and for evaluating prognostic significance.[20]

In this study, the BRCA1 cases or the BRCA2 cases were significantly different compared with the non-matched control group in terms of age, tumor grade, mitotic score, ER positivity, PR positivity, topo II-alpha staining, Mib1 statining, p53 immunopositivity, and microvessel density (Tables 2, 3). The only significant difference between the BRCA1 and BRCA2 groups was found for tumor grade ($P < .05$) with high-grade tumors observed for 64% of BRCA1 cases and 23% of BRCA2 cases (Table 2).

In confirmation of previous data, we found that the age of onset in patients with HBC is roughly a decade earlier than in patients with non-HBC.[7,9] The frequency of medullary and lobular features in BRCA1- and BRCA2-related breast cancer observed here is consistent with previous reports.[7,10] In addition, patients with HBC generally have neoplasms that show adverse histological features. These include tumors with high nuclear grade, high proliferation indices, lack of hormone receptor positivity, and an increase incidence of p53 immunopositivity.[6,7,10,12,13] In spite of these negative prognostic markers, other investigators have shown that patients with HBC have comparable or improved survival compared with patients with non-HBC.[6-9,24,25] In a larger study that included 30 BRCA1 patients and 20 BRCA2 patients, we evaluated overall survival compared with sporadic controls matched for tumor size, age, and date of diagnosis, and there were no differences in survival at 5 or 10 years.[24] Thus, our data suggest that survival is similar for BRCA1 patients, BRCA2 patients, and non-HBC patients.

In one report of patients with BRCA1-related breast cancers, grade was believed to segregate as a genetic trait within families. Moreover, this was attributed to mitotic index segregation, and a possible genotype-phenotype correlation was suggested. Although our patient numbers are small, especially when evaluated per kindred (Table 1), our data do not confirm this hypothesis. A normal range was observed for all evaluated parameters within families, including grade, mitotic index, Mib1, topo II-alpha, and p53.

To understand this apparent paradox between histological findings and clinical course, we investigated the expression of several markers, which have not previously been evaluated in HBC. Amplification of HER2/neu oncogene has been correlated with more aggressive disease. The number of cases in our study that showed increased expression of this oncogene was too small to yield statistically significant results.

Microvessel density has also been suggested to yield prognostic information in breast cancer. Low microvessel density suggests a more favorable clinical course.[15,22] Interestingly, we found that the average microvessel density score in tumors from patients with BRCA1-related breast cancer was statistically lower than that observed in a control group. The prognostic implications of microvessel density and its reproducibility is controversial. It is possible that tumors arising in patients with HBC may have a decreased ability to undergo angiogenesis compared with non-HBC tumors, and this may modify the clinical course. Further studies will be required to explore this observation.

Our proliferation data suggest another possible molecular mechanism that may partially explain the clinical response of patients with HBC to therapy. It has been suggested previously that breast cancers with a high population of cycling cells have a high likelihood of responding to chemotherapy.[26] However, those tumors that do not initially respond or in which a large number of cells are not killed, would show an early relapse.[26] Thus, the proliferation index of a breast cancer could be viewed as showing both positive and negative clinical correlations. We found, as others also have, that tumors from patients with HBC have higher proliferation indices than tumors from patients with non-HBC.[7,8,10] In addition, we have shown in this study that these high proliferation indices correlate with increased expression of topo II-alpha. Topo II-alpha is an enzyme elevated in proliferating cells, where its function is to separate intertwined DNA strands before mitosis. Although clearly a marker of cell proliferation, topo II-alpha is also the molecular target of many clinically used antitumor drugs.[14] Cells that express high topo II-alpha levels are drug sensitive, and cells that express low topo II-alpha are drug resistant. Some of the drugs that target topo II-alpha such as doxorubicin are used in the treatment of breast cancer. It is possible that the increased expression of topo II-alpha in HBC might play an important role in the relatively favorable clinical response of these patients to chemotherapy. If resistant clones do not arise early in the course of HBC, then the high proliferative indices in HBC tumors could have positive prognostic implications. The decreased level of angiogenesis in BRCA1-related tumors may reduce the rate of early metastatic spread of tumor cells. The positive prognostic implications of high tumor cell proliferation and decreased angiogenesis might balance out negative indicators found in this group of tumors such as increased frequency of p53 immunopositivity, high nuclear grade, and lack of hormone receptor positivity. Thus, it is plausible mechanistically that patients with HBC may have a similar clinical outcome to patients with non-HBC. Further work correlating therapy and clinical outcome with molecular markers in HBC and non-HBC would be useful to answer this question.

Acknowledgment. The authors thank the many families who participated in the research studies.

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ORAL CONTRACEPTIVES AND THE RISK OF HEREDITARY OVARIAN CANCER

STEVEN A. NAROD, M.D., HARVEY RISCH, M.D., PH.D., ROXANA MOSLEHI, M.SC., ANNE DØRUM, M.D., SUSAN NEUHAUSEN, PH.D., HAKAN OLSSON, M.D., DIANE PROVENCHER, M.D., PAOLO RADICE, PH.D., GARETH EVANS, M.D., SUSAN BISHOP, M.SC., JEAN-SEBASTIEN BRUNET, M.SC., AND BRUCE A.J. PONDER, M.D., PH.D., FOR THE HEREDITARY OVARIAN CANCER CLINICAL STUDY GROUP*

ABSTRACT

Background Women with mutations in either the *BRCA1* or the *BRCA2* gene have a high lifetime risk of ovarian cancer. Oral contraceptives protect against ovarian cancer in general, but it is not known whether they also protect against hereditary forms of ovarian cancer.

Methods We enrolled 207 women with hereditary ovarian cancer and 161 of their sisters as controls in a case–control study. All the patients carried a pathogenic mutation in either *BRCA1* (179 women) or *BRCA2* (28 women). The control women were enrolled regardless of whether or not they had either mutation. Lifetime histories of oral-contraceptive use were obtained by interview or by written questionnaire and were compared between patients and control women, after adjustment for year of birth and parity.

Results The adjusted odds ratio for ovarian cancer associated with any past use of oral contraceptives was 0.5 (95 percent confidence interval, 0.3 to 0.8). The risk decreased with increasing duration of use (*P* for trend, <0.001); use for six or more years was associated with a 60 percent reduction in risk. Oral-contraceptive use protected against ovarian cancer both for carriers of the *BRCA1* mutation (odds ratio, 0.5; 95 percent confidence interval, 0.3 to 0.9) and for carriers of the *BRCA2* mutation (odds ratio, 0.4; 95 percent confidence interval, 0.2 to 1.1).

Conclusions Oral-contraceptive use may reduce the risk of ovarian cancer in women with pathogenic mutations in the *BRCA1* or *BRCA2* gene. (N Engl J Med 1998;339:424-8.)

APPROXIMATELY 10 percent of cases of invasive epithelial ovarian cancer are hereditary, occurring predominantly in women with germ-line mutations in the *BRCA1* or the *BRCA2* gene (unpublished data). The lifetime risk of ovarian cancer is approximately 45 percent among women with *BRCA1* mutations and 25 percent among those with *BRCA2* mutations.[1-3]

Current strategies for reducing the risk of ovarian cancer in women carrying *BRCA1* or *BRCA2* mutations include prophylactic oophorectomy and ultrasound screening, but the extent of risk reduction associated with either of these procedures is not known.[4] A third potential strategy is chemoprevention. The risk of ovarian cancer is reduced by 50 percent or more in unselected women with long-term use of oral contraceptives.[5,6] An oral contraceptive agent is appealing as a possible preventive treatment, because these agents are well tolerated and their side effects are known. To evaluate the potential benefit of oral-contraceptive use in women at high risk for ovarian cancer, we studied 207 patients with *BRCA1* or *BRCA2* mutations and ovarian cancer and 161 of their sisters, who served as controls.

METHODS

Subjects

The patients were 207 women born between 1925 and 1960 in whom invasive epithelial ovarian cancer had been diagnosed and who were found by molecular testing to carry a germ-line mutation in either the *BRCA1* or the *BRCA2* gene (Table 1). They were identified in three ways. Sixteen were women in whom ovarian cancer had been diagnosed in Ontario, Canada, after January 1, 1995. Twenty-six were Ashkenazi Jewish women with a history of ovarian cancer who were identified from the gynecology–oncology records of 11 hospitals in North America. One hundred sixty-five were women identified by members of the Breast Cancer Linkage Consortium: 37 from the United Kingdom, 39 from other European countries, 67 from the United States, and 22 from Canada. The women's average ages at the time of diagnosis in the three groups were 49, 52, and 49 years, respectively.

All living sisters of the patients who were born between 1925 and 1960 were eligible to be control subjects. The use of these women as controls ensured that the geographic and ethnic characteristics of the patients and the control women would be similar. Furthermore, the sisters had the same a priori familial risk of ovarian cancer as the patients; that is, before the diagnosis of breast or ovarian cancer in the latter, both a patient and her sister would have had the same risk of ovarian cancer on the basis of family history alone. Fifty-one of the patients had no sisters, and no information on family history was available for another 12 pa-

From the Centre for Research on Women's Health, Women's College Hospital, University of Toronto, Toronto (S.A.N., S.B., J.-S.B.); the Department of Epidemiology and Public Health, Yale University, New Haven, Conn. (H.R.); the Department of Medical Genetics, University of British Columbia, Vancouver, Canada (R.M.); the Unit of Medical Genetics, Norwegian Radium Hospital, Oslo, Norway (A.D.); the Department of Medical Informatics, University of Utah, Salt Lake City (S.N.); the Department of Oncology, Lund University, Lund, Sweden (H.O.); the Department of Obstetrics and Gynecology, Notre Dame Hospital, Montreal (D.P.); the Istituto Nazionale Tumori, Milan, Italy (P.R.); the Department of Medical Genetics, St. Mary's Hospital, Manchester, United Kingdom (G.E.); and the Cancer Research Campaign Genetic Epidemiology Unit, Cambridge University, Cambridge, United Kingdom (B.A.J.P.). Address reprint requests to Dr. Narod at the Centre for Research on Women's Health, Women's College Hospital, 790 Bay St., Rm. 750a, Toronto, ON M5G 1N8, Canada.

Jan G.M. Klijn, M.D. (Department of Medical Oncology, Rotterdam Cancer Institute, Rotterdam, the Netherlands), was also an author.

*Other members of the study group are listed in the Appendix.

TABLE 1. CHARACTERISTICS OF PATIENTS WITH OVARIAN CANCER AND CONTROL WOMEN.*

CHARACTERISTIC	PATIENTS WITH OVARIAN CANCER (N=207)	CONTROL WOMEN (N=161)
Age (yr)	54±8	52±8
Residence (%)		
United States	44	38
Canada	19	29
Europe	37	33
Race or ethnic group (%)		
Ashkenazi Jewish	26	10
Other white	68	88
Black	2	2
Unknown	3	1
Parity (%)		
0	14	15
1	9	13
≥2	77	72
Mean age at first childbirth (yr)	24	24
Mean age at last childbirth (yr)	29	29
Any use of oral contraceptives (%)	50	70†
Duration of oral-contraceptive use (yr)	4±5	6±5‡
Age at beginning of oral-contraceptive use (yr)	24±5	22±5

*Plus–minus values are means ±SD. Because of rounding, percentages do not always total 100.

†$P<0.001$ for the comparison with the women with ovarian cancer.

‡$P=0.01$ for the comparison with the women with ovarian cancer.

tients. The remaining 144 patients had a total of 328 sisters. Of these, 167 sisters were not studied because they had died, had ovarian cancer (these women were invited to be study patients), were born before 1925 or after 1960, were too ill to respond, or were unwilling to be questioned. Thus, there were 161 control women, or 0.8 per patient (range, 0 to 7).

Data on mutations were available for 95 of the 161 control women. According to molecular testing, 53 were carriers of the same *BRCA1* or *BRCA2* mutations as their sisters, and 42 were not carriers. Ideally, the group of control women would have been restricted to mutation-positive women who did not have ovarian cancer and had not undergone oophorectomy at the age at which ovarian cancer was diagnosed in their sisters. However, this approach was impractical, because there were only 42 unaffected mutation-positive sisters who had both ovaries at the time of the diagnosis of ovarian cancer in their sisters. Women with a history of breast cancer were not excluded from the study; 63 of the patients (30 percent) and 29 of the control women (18 percent) had a previous diagnosis of breast cancer.

Analysis of Mutations

Mutation analysis was performed by several established detection techniques, and all mutations were confirmed by direct sequencing of DNA samples. For the women in Ontario, DNA samples were screened by the protein-truncation test for mutations in exons 10 and 11 of *BRCA1* and exon 11 of *BRCA2*.[7] The Ashkenazi Jewish women were screened for three founder mutations, two in *BRCA1* (185delAG and 5382insC) and one in *BRCA2* (6174delT).[8] The women in the group identified by the Breast Cancer Linkage Consortium were screened for mutations by techniques routinely used in the participating laboratories. These techniques included direct sequencing of DNA, heteroduplex analysis, single-strand conformation analysis, and allele-specific oligonucleotide hybridization. In every woman, the actual sequence variant in *BRCA1* or *BRCA2* was established by direct sequencing of DNA.

Study Protocol

The patients and control women were asked about their reproductive histories, methods of contraception (including use of oral contraceptive agents and tubal ligation), and history with respect to oophorectomy. The women were asked at what age they first took an oral contraceptive, at what age they stopped, and the total duration of oral-contraceptive use. No information was requested about the specific oral contraceptive agent taken. Two patients had undergone unilateral oophorectomy for a benign condition before the diagnosis of ovarian cancer. Among the control women, 72 had undergone oophorectomy (5 unilateral and 67 bilateral). The average age at the time of bilateral oophorectomy was 45 years.

Statistical Analysis

The mean duration of oral-contraceptive use in the patients and control women was compared by the nonparametric Wilcoxon two-sample test. Odds ratios were estimated by unconditional logistic-regression analysis with control for other covariates, including geographic area of residence (United States, Canada, United Kingdom, or elsewhere in Europe), year of birth, parity, and age at delivery of a first child. The last three variables were included as continuous terms.

To control for possible confounding effects of ethnic group, a separate matched analysis was performed. Patients were matched with their sisters, and conditional logistic regression for matched sets (with variable ratios of patients to controls) was performed. This analysis was based on 89 case–control pairs, because patients with no sisters were excluded. All statistical tests were two-sided.

RESULTS

The characteristics of the 207 patients with ovarian cancer and the 161 controls were similar (Table 1). Fifty percent of the patients and 70 percent of the control women reported a history of oral-contraceptive use ($P<0.001$). The average duration of oral-contraceptive use for the patients was four years, significantly less than the average duration for the control women (six years; $P=0.01$).

Among the 207 patients, 179 had *BRCA1* mutations and 28 had *BRCA2* mutations. Among the 161 control women, 53 were known to have mutations (50 had *BRCA1* mutations and 3 had *BRCA2* mutations) and 42 were known to be noncarriers; the remaining 66 were not tested. In these three subgroups of women, a history of oral-contraceptive use was reported by 77 percent, 64 percent, and 67 percent, respectively (as compared with 50 percent of the patients). The average duration of use was also greater for each of the three subgroups of control women (five, five, and seven years, respectively) than for the patients (four years). Because the duration of oral-contraceptive use was similar in the three subgroups of control women, and because of the small sizes of those subgroups, they were combined for most of the multivariate analyses. The pattern of oral-contraceptive use did not differ significantly between the control women who had undergone bilateral oophorectomy (69 percent; mean duration of use,

TABLE 2. ASSOCIATION BETWEEN ORAL-CONTRACEPTIVE USE AND RISK OF OVARIAN CANCER.*

VARIABLE	ALL CONTROL WOMEN		CONTROL WOMEN WITH *BRCA1* OR *BRCA2* ONLY†
	UNIVARIATE ANALYSIS	MULTIVARIATE ANALYSIS	MULTIVARIATE ANALYSIS
	odds ratio (95% CI)		
Any use vs. none	0.4 (0.3–0.7)	0.5 (0.3–0.8)	0.4 (0.2–0.7)
Duration of use (yr)			
0	1.0	1.0	1.0
<3	0.7 (0.4–1.2)	0.8 (0.4–1.4)	0.4 (0.3–0.9)
3 to <6	0.4 (0.2–0.7)	0.4 (0.2–0.9)	0.4 (0.1–1.0
≥6	0.3 (0.2–0.6)	0.4 (0.2–0.7)	0.3 (0.1–0.7)
Trend per year of use	0.9 (0.9–1.0)	0.9 (0.9–1.0)	0.9 (0.9–1.0)

*CI denotes confidence interval. Multivariate odds ratios have been adjusted for year of birth, parity, age at the delivery of a first child, and geographic area of residence. Multivariate analyses of carriers of a *BRCA1* or *BRCA2* mutation have also been adjusted for the mutation type (*BRCA1* or *BRCA2*). Each oral-contraceptive variable was considered in a separate model.

†This analysis includes only the 53 control women who were confirmed carriers of mutations.

five years) and those with both ovaries (72 percent; mean duration of use, six years; $P=0.37$).

The odds ratios for ovarian cancer associated with oral-contraceptive use according to unconditional logistic-regression analysis are shown in Table 2. The risk of ovarian cancer decreased with the duration of use (multivariate P for trend, <0.001). Women who took an oral contraceptive agent for six or more years had a reduction in risk of 60 percent. The reduction in risk was similar for carriers of the *BRCA1* and *BRCA2* mutations. The odds ratio for carriers of the *BRCA1* mutation who had used oral contraceptives, as compared with those who had not, was 0.5 (95 percent confidence interval, 0.3 to 0.9), and that for carriers of the *BRCA2* mutation was 0.4 (95 percent confidence interval, 0.2 to 1.1). Adjustments for parity, age at the delivery of a first child, and age at the delivery of a last child did not significantly change the magnitude of the odds-ratio estimates associated with oral-contraceptive use.

The results of the matched analysis were very similar to those shown in Table 2. The odds ratios for ovarian cancer were 0.9 (95 percent confidence interval, 0.4 to 2.1), 0.4 (95 percent confidence interval, 0.2 to 1.2), and 0.3 (95 percent confidence interval, 0.1 to 0.7) for oral-contraceptive use for less than three years, three to less than six years, and six or more years, respectively ($P=0.02$).

Among the 63 patients who had had breast cancer in addition to ovarian cancer, the average duration of oral-contraceptive use was five years, as compared with four years among those who had not had breast cancer. The average duration of oral-contraceptive use was six years among the 29 control women who had had breast cancer and among those who had not had breast cancer.

DISCUSSION

In this multicenter case–control study, the use of oral contraceptives was associated with a significant reduction in the risk of ovarian cancer among women with a mutation in the *BRCA1* or *BRCA2* gene. The reduction in risk was 20 percent for up to three years of use, rising to 60 percent for six or more years of use.

The magnitude of the protective effect of oral contraceptives in carriers of *BRCA1* and *BRCA2* mutations is consistent with that previously found in the general population. In a meta-analysis of 12 case–control studies of oral-contraceptive use and the risk of ovarian cancer in the United States,[9] the risk decreased with increasing length of oral-contraceptive use. In the six population-based studies, the risk reduction was 34 percent for those who had ever used oral contraceptives and 70 percent for those with six or more years of use. In the six hospital-based studies, the corresponding risk reductions were 30 percent and 45 percent, respectively.

The strengths of the present study are that the cases of ovarian cancer were identified through a large international consortium, all patients with ovarian cancer were confirmed carriers of mutations, and the control group consisted of sisters of the patients. We think the smaller size of the control group is counterbalanced by the similarity of the control women to the patients, because by definition they shared the same family history, were members of the same ethnic group, and were from the same geographic region.

Ashkenazi Jewish women were somewhat overrepresented among the patients and French-Canadian women among the controls. These differences reflect the average family size of women from the two ethnic groups, rather than the willingness of the sis-

ters of the patients to participate. On average, a Jewish woman had 1.2 sisters, and a French-Canadian woman had 4.3 sisters. In other respects, the patients and control women were well matched.

The ideal control group for this study might be sisters of the patients who still had both their ovaries and who carried the same mutation but in whom ovarian cancer had not developed by the age at which it was diagnosed in their sisters. Unfortunately, we could not identify sufficient numbers of control women with these characteristics, and we therefore extended the control group to include all unaffected living sisters of the patients. Nevertheless, more of the sisters with and without mutations in *BRCA1* or *BRCA2* than patients had used oral contraceptives. The extent of misclassification introduced by the inclusion of sisters without mutations is likely to be minimal, given the similarity of the results of the analysis based on all control women and on only sisters with mutations. Furthermore, if the use of oral contraceptives protects against ovarian cancer, then a higher proportion of women with mutations who did not have ovarian cancer would have been expected to have taken oral contraceptives. This was true: 77 percent of the control women with mutations had taken oral contraceptives, as compared with 64 percent of those without mutations.

Our study included control women who had undergone oophorectomy. Fewer of these women might have taken oral contraceptives than expected if the oophorectomy was performed before menopause. However, there was little difference in the frequency of use of oral contraceptives between control women who had had their ovaries removed and those who had not. Selection bias of this type should lead to underestimation of the magnitude of the risk reduction associated with oral-contraceptive use.

Adjustment for parity, the presence or absence of tubal ligation, and ages at the delivery of a first and last child did not influence the protective effect of oral-contraceptive use. Increasing parity appears to be protective against hereditary ovarian cancer, as it is for ovarian cancer in the general population.[9] There are no other known risk factors for ovarian cancer that are likely to have been confounders in the present study.

A limitation of this study is that it included only living women with ovarian cancer as case patients. This was true because of the difficulty of ascertaining whether deceased patients had carried mutations and of obtaining an accurate history of contraceptive use in interviews with surrogates. If oral-contraceptive use is associated with a higher case fatality rate for ovarian cancer, then this selection strategy will exaggerate the protective effect of oral contraceptives. On average, the women in our study stopped using oral contraceptives 17 years before the diagnosis of ovarian cancer, and only 12 women had taken an oral contraceptive agent during the 5-year period before diagnosis.

It is important to establish whether the risk of breast cancer in women with *BRCA1* or *BRCA2* mutations is influenced by oral-contraceptive use, especially if oral contraceptives are to be recommended to healthy carriers as chemopreventive agents. Oral-contraceptive use has been associated with a small increase in the risk of breast cancer in young[9] and older[10] women. In a large meta-analysis, current use of oral contraceptives was associated with a relative risk of 1.2 for breast cancer, and past use was associated with a relative risk of 1.1. However, there was no increased risk in the subgroup of women with a family history of breast cancer (defined as having a mother or sister affected). In one study of Jewish women with breast cancer, the frequency of long-term oral-contraceptive use was higher among women who had a *BRCA1* or *BRCA2* mutation than among women without a mutation.[11] We found no difference in the history of oral-contraceptive use between women who had had breast cancer and those who had not, but our study was not specifically designed to evaluate this issue.

Our data suggest that the administration of an oral contraceptive agent should be considered as part of a program of prevention for women with *BRCA1* or *BRCA2* mutations who have not had ovarian cancer. However, our data do not allow us to address the specific formulation to be recommended or the age at which treatment should begin.

Supported by grants from the National Institutes of Health (1R01 CA63682, CA63678, CA57601, CA61231, and CA55914), the National Cancer Institute of Canada Breast Cancer Initiative, the Department of the Army (DAMD17-94-J-4299 and DAMD 17-94-J-4260), the Canadian Genetic Diseases Network, the Canadian Breast Cancer Foundation (Ontario chapter), the Fonds de la Recherche en Sante du Quebec, the Women's Cancer Program of the Dana–Farber Cancer Institute, and the Norwegian and Dutch Cancer Societies.

APPENDIX

Other members of the Hereditary Ovarian Cancer Clinical Study Group are as follows: Montreal: W. Foulkes, P. Tonin, J. Rosenblatt, P. Ghadirian, C. Perret, A.-M. Mes-Masson, and B. Godard; Toronto: B. Rosen, D. Cole, J. McLaughlin, J. Murphy, L. Bradley, I. Fan, J. Abrahamson, and E. Warner; Philadelphia: T. Rebbeck, B. Weber, F. Couch, M. Daly, A. Godwin, and J. Wagner-Costalos; Washington, D.C.: C. Lerman and B. Peshkin; Durham, N.C.: A. Futreal and J. Lancaster; Chicago: O. Olopade and S. Cummings; Salt Lake City: L. Cannon-Albright and L. Steele; Boston: J. Garber and N. Tung; Omaha, Neb.: H. Lynch, J. Lynch, C. Snyder, and C. Durham; Los Angeles: B. Karlan; New Hyde Park, N.Y.: D. Smotkin; New York: A. Fields, D. Russo, and K. Antman; Cambridge, United Kingdom: D. Ford and D. Easton; Lyons, France: G. Lenoir, O. Serova, and S. Mazoyer; Rotterdam, the Netherlands: E. Meijers-Heijboer and L. Verhoog; Manchester, United Kingdom: F. Lalloo, Lund, Sweden: O. Johannson and A. Borg; and Oslo, Norway: P. Moller.

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Haplotype and Phenotype Analysis of Nine Recurrent *BRCA2* Mutations in 111 Families: Results of an International Study

Susan L. Neuhausen,[1] Andrew K. Godwin,[2] Ruth Gershoni-Baruch,[4] Elizabeth Schubert,[5] Judy Garber,[6] Dominique Stoppa-Lyonnet,[7] Edith Olah,[8] Bela Csokay,[8] Olga Serova,[9] Fiona Lalloo,[10] Ana Osorio,[11] Michael Stratton,[12] Kenneth Offit,[13] Jeff Boyd,[14] M. Adelaide Caligo,[15] Rodney J. Scott,[16,*] Andy Schofield,[17] Eric Teugels,[18] Manfred Schwab,[19] Lisa Cannon-Albright,[1] Timothy Bishop,[20] Douglas Easton,[21] Javier Benitez,[11] Mary-Claire King,[5] Bruce A. J. Ponder,[22] Barbara Weber,[3] Peter Devilee,[23] Åke Borg,[24] Steven A. Narod,[25] and David Goldgar[10]

[1]Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City; [2] Department of Medical Oncology, Fox Chase Cancer Center, and [3]Department of Hematology-Oncology, University of Pennsylvania, Philadelphia; [4]Department of Human Genetics, Rambam Medical Center, Haifa, Israel; [5] Departments of Medicine and Genetics, University of Washington, Seattle; [6]Unite de Génétique Oncologique, Institute Curie, Paris; [7]Dana Farber Cancer Institute, Boston; [8]National Institute of Oncology, Budapest; [9] International Agency for Research on Cancer, Lyon; [10]University Department of Medical Genetics and Regional Genetic Service, St. Mary's Hospital, Manchester, United Kingdom; [11]Department of Genetics, Fundación Jimenez Díaz, Madrid; [12]Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, United Kingdom; Departments of [13]Human Genetics and [14]Surgery, Memorial Sloan-Kettering Cancer Center, New York; [15]Department of Oncology, University of Pisa, Pisa; [16]Department of Human Genetics, Kantonsspital Basel, Basel; [17]Department of Medical Genetics, University of Aberdeen Medical School, Aberdeen; [18]Oncology Center, Free University of Brussels; [19]German Cancer Research Center, Heidelberg; [20]Imperial Cancer Research Fund Genetic Epidemiology Laboratory, Leeds; [21] Cancer Research Campaign (CRC) Genetic Epidemiology Unit, Institute of Public Health, and [22]CRC Human Cancer Genetics Group, Department of Oncology, University of Cambridge, Cambridge; [23]Department of Human Genetics, University of Leiden, Leiden; [24]Department of Oncology, University Hospital, Lund, Sweden; and [25]Centre for Research in Women's Health, University of Toronto, Toronto

Summary

Several *BRCA2* mutations are found to occur in geographically diverse breast and ovarian cancer families. To investigate both mutation origin and mutation-specific phenotypes due to *BRCA2*, we constructed a haplotype of 10 polymorphic short tandem-repeat (STR) markers flanking the *BRCA2* locus, in a set of 111 breast or breast/ovarian cancer families selected for having one of nine recurrent *BRCA2* mutations. Six of the individual mutations are estimated to have arisen 400–2,000 years ago. In particular, the 6174delT mutation, found in ~1% of individuals of Ashkenazi Jewish ancestry, was estimated to have arisen 29 generations ago (1-LOD support interval 22–38). This is substantially more recent than the estimated age of the *BRCA1* 185delAG mutation (46 generations), derived from our analogous study of *BRCA1* mutations. In general, there was no evidence of multiple origins of identical *BRCA2* mutations. Our study data were consistent with the previous report of a higher incidence of ovarian cancer in families with mutations in a 3.3-kb region of exon 11 (the ovarian cancer cluster region [OCCR]) ($P = .10$); but that higher incidence was not statistically significant. There was significant evidence that age at diagnosis of breast cancer varied by mutation ($P < .001$), although only 8% of the variance in age at diagnosis could be explained by the specific mutation, and there was no evidence of family-specific effects. When the age at diagnosis of the breast cancer cases was examined by OCCR, cases associated with mutations in the OCCR had a significantly older mean age at diagnosis than was seen in those outside this region (48 years vs. 42 years; $P = .0005$).

Introduction

The isolation of *BRCA1* (Miki et al. 1994) and *BRCA2* (Wooster et al. 1995; Tavtigian et al. 1996), two genes predisposing to early-onset breast cancer and ovarian cancer, has resulted in rapid identification of a large number of families with mutations in these genes (Breast Cancer Information Core) (Couch et al. 1996*b*; Szabo and King 1997). Although both genes exhibit a large number of distinct mutations, several mutations have been found to recur in a number of independently as-

Received February 13, 1998; accepted for publication April 10, 1998; electronically published May 8, 1998.

Address for correspondence and reprints: Dr. Susan L. Neuhausen, Division of Genetic Epidemiology, Department of Medical Informatics, 391 Chipeta Way, Suite D2, Salt Lake City, UT 84108. E-mail: susan@episun3.med.utah.edu

* Present affiliation: Hunter Area Pathology Service, John Hunter Hospital, University of Newcastle, Newcastle, Australia.

0002-9297/98/6206-0015$02.00

certained families of apparently diverse geographical origin, as well as in families largely confined to a single population.

Genes responsible for inherited cancer, like many other disease genes, have been associated with a wide diversity of expression. This is seen not only in variability in the age at diagnosis of cancer but also in the anatomical site at which the tumor originates. More important, at least from the clinical perspective, is the degree to which specific mutations and accompanying genetic backgrounds influence the expression of *BRCA2* in terms of site and age at diagnosis. For *BRCA2*, Gayther et al. (1997) have provided evidence that mutations in an ~3.3-kb nucleotide region of exon 11 (denoted the "ovarian cancer-cluster region" [OCCR]) are associated with a higher incidence of ovarian cancer relative to breast cancer. In that study, this was highly significant, with an ovarian:breast cancer ratio of 11:45 inside, and 22:282 outside, the OCCR. In the present studies, four of the mutations examined were within the OCCR, whereas the other five were outside this region. This allowed us to examine, with the present data set, the OCCR hypothesis.

In a previous paper (Neuhausen et al. 1996*b*), we analyzed six recurrent *BRCA1* mutations for haplotype conservation, over a 3-Mb segment containing the *BRCA1* gene, using nine STR markers. We also investigated the relationship between the position of the mutation and the phenotype (in terms of both age at diagnosis of breast cancer and proportion of ovarian cancer) of the families carrying each mutation. In the present article, we have undertaken a similar study of recurrent *BRCA2* mutations, addressing both mutation origin and the relationship between mutation and phenotype. To do this, we constructed a haplotype of 10 polymorphic STR markers flanking the *BRCA2* locus in a set of 111 families (selected to contain one of nine *BRCA2* mutations that had been identified a minimum of three times) and analyzed the phenotype associated with each mutation. For five mutations for which sufficient haplotype data existed, we estimated the age of the mutation, using a modified version of our mathematical model developed for our *BRCA1* analysis.

Subjects and Methods

Family Ascertainment

Families with one of the nine mutations were from 24 centers located in 13 countries in Europe and North America. The families had been previously ascertained for a variety of reasons, including research studies, directed screening of case series of ovarian or male breast cancer, or attendence at a cancer genetics clinic. Appropriate informed consent was obtained from all participants. When possible, pedigree information was obtained, although, for several centers, no such history was available and, for other centers, only a limited family history could be obtained. All cases of breast and ovarian cancer reported in the pedigree were included in the study, with the exception of cases who were known to not carry the *BRCA2* mutation segregating in the family. No independent verification of diagnosis was obtained, and, for a small proportion of cases, age at diagnosis was not available.

Samples for the 982del4 mutation were from the United States and France; those for 2041insA, from Germany, Canada, and the United States; those for 3034del4, from Belgium, Canada, Spain, France, Switzerland, Italy, and the United States; those for 4486delG, from Sweden; those for 5573insA, from the Netherlands; those for 6174delT, from Canada, France, Israel, Hungary, Sweden, the United Kingdom, and the United States; those for 6503delTT, from Belgium, the Netherlands, Sweden, and the United Kingdom; those for 9254del5, from France and Spain; and, those for 9326insA, from Hungary, Sweden, and the United Kingdom.

Genotyping of 13q Markers

Genotyping was performed at four centers. The families collected by the University of Washington in Seattle, the National Institute of Oncology in Budapest, and the Fundacion Jimenez Diaz in Madrid were genotyped in their respective laboratories; all other families were genotyped in the Genetic Epidemiology Laboratory at the University of Utah. At all centers, the same five DNA samples were used as controls, and a similar protocol was followed. All 10 markers genotyped were STR loci assayed by PCR, with standard procedures. All the results in the tables are from analyses of all 10 markers. For all mutations except 6174delT, allele frequencies used in the likelihood calculations were as reported in Genome Database, from typings of ~80 independent CEPH chromosomes. For analysis of family samples of Ashkenazi Jewish ancestry carrying the 6174delT mutation, we estimated marker-allele frequencies from the haplotype data of the non–mutation-bearing chromosomes. In all cases, allele sizes were matched according to the genotype of CEPH reference individual 1347-02, who was used as a control on each gel. The genetic map assumed for the haplotype analyses was derived from physical-mapping data (Couch et al. 1996*a*; S. L. Neuhausen, unpublished data), under the assumption that 1.5 cM = 1 Mb. Note that this rate is higher than the usual 1:1 ratio assumed as a genomewide average; this was done to ensure that the total distance of the map was in agreement with that of the published genetic map (Dib et al. 1996). None of the markers were located intragenic to *BRCA2*. The assumed map order and dis-

Table 1

Summary of STRs Used in Haplotype Analysis

Marker	Position (cM)	No. of Alleles	Heterozygosity[a] (%)	Size (Frequency) of Common Allele (bp)	Genotype of 1347-02 (bp)
D13S290	2.70	6	46	176 (.71), 190 (.11), 188 (.11)	190/176
D13S1444	1.35	9	80	167 (.41), 169 (.24), 177 (.11)	177/167
D13S1700	1.20	18	89	308 (.12), 312 (.09), 258 (.09)	320/254
D13S260	1.00	9	78	163 (.41), 161 (.13), 171 (.09)	163/161
D13S1699	.72	6	67	150 (.54), 146 (.37)	156/146
D13S1698	.63	10	63	152 (.35), 154 (.30)	168/160
BRCA2	.0				
D13S171	−.60	6	72	241 (.32), 231 (.32), 227 (.25)	231/231
D13S1695	−.96	11	79	245 (.37), 247 (.23)	249/235
D13S310	−2.10	5	70	146 (.40), 144 (.24), 140 (.24)	146/146
D13S267	−3.12	6	69	148 (.44), 160 (.29), 154 (.17)	160/148

[a] Determined from genotyping of 80–100 chromosomes.

tances and the descriptions of the markers used are given in table 1.

When possible, haplotypes associated with each mutation were inferred from multiple samples of related individuals within each kindred known to have the same mutation; otherwise, multilocus genotypes were compared. When haplotypes could not be determined with certainty, all possible haplotypes (to a maximum of 64) consistent with the observed multilocus genotypes were considered in the likelihood analysis, in a manner analogous to the phase calculations in multipoint linkage analysis.

Analysis of Haplotype Data

The estimation of the age of the mutations was performed by use of the same statistical model that had been used in our previous analysis of *BRCA1* (Neuhausen et al. 1996*b*), with several minor modifications. In brief, the joint likelihood of the *BRCA2* haplotypes (or all possible haplotypes from families with a given mutation, relative to a presumed ancestral haplotype) is written as a function of the recombination fraction between the disease and each marker; the number of generations, G, since the mutation arose; and the mutation rate and allele frequencies at each marker locus. The marker D13S1700 was assumed to have a higher mutation rate (.01) than the other markers (.002 for a tetranucleotide repeat and .0006 for a dinucleotide repeat), on the basis of both the large number of alleles and the observation of mutations within families. We also included another parameter, μ_D, the proportion of families with an independent mutation identical to that of the presumed ancestral haplotype. This parameter is analogous to genetic heterogeneity in standard linkage analysis and can be estimated from the data.

The method of maximum likelihood was used to find the value of G that, among families with identical mutations, best fitted the pattern of haplotype sharing at the 10 marker loci. Approximate support intervals for the age of each mutation were calculated by finding the value of G on either side of the most likely value that had a $\geq$10-fold decrease in likelihood. A test for heterogeneity of mutation origin was performed by comparing the likelihood at the maximum-likelihood estimates of G and μ_D with the analogous likelihood, assuming $\mu_D = 0$. Each generation is estimated to be 20 years.

Analysis of Phenotype Data

For each mutation, the number of families with that mutation, the number of female and male breast cancer cases, and the number of ovarian cancer cases were tabulated. To partially counter any effects of ascertainment of those directed-screening cases of breast and ovarian cancers, we also examined the data only in those families in which there were at least three cases of cancer, where a case is defined as a female breast cancer at age <60 years, an ovarian cancer, or a male breast cancer. In this second tabulation, only cases of female breast cancer at age <60 years were counted in the breast cancer results; this was done in order to increase the probability that they were associated with the *BRCA2* mutation segregating in the family.

To test for heterogeneity, in the proportion of affected individuals who had ovarian cancer, as a function of whether the mutation associated with a given family was inside or outside the hypothesized OCCR, a randomization test was performed. Specifically, random permutations of families with the nine mutations were performed, in which the number of families with each mutation was kept equal to that present in the actual data set. After this permutation step, the mutations were grouped according to their location respective to the OCCR. Each such permutation resulted in a different

Table 2

Results of Haplotype Analysis of Nine Mutations

Mutation	No. of Families[a]	No. of Countries[b]	Core Haplotype at					Consistency Index[c]	G	1-LOD Interval
			D13S260	D13S1699	D13S1698	D13S171	D13S1695			
982del4	5 (3/2)	2	161	146	154	231	253	5/5	18	(4–43)
2034insA	5 (3/2)	3	163	150	166	241	247	3/5	36	(13–64)
3034del4	11 (4/7)	7	163	146	154	227	245	2/11	80	(46–134)
4484delG	4 (0/4)	1	169	156	166	241	247	4/4	Not calculated	
5573insA	3 (3/0)	1	165	146	154	227	245	2/3	Not calculated	
6174delT	69 (22/47)	7	161	146	152	239	251	45/69	29	(22–38)
6503delTT	7 (5/2)	4	163	150	158	227	245	3/5	52	(24–98)
9254del5	3 (2/1)	2	163	150	154	231	X	2/3	Not calculated	
9326insA	4 (1/3)	3	171	152	152	231	245	2/4	Not calculated	

[a] Data in parentheses are number of families in which haplotypes could be determined/number of families for which only multilocus genotype data were available.

[b] For names of countries, see the Subjects and Methods section.

[c] Number of samples/families consistent with core haplotype for all five markers listed.

2×2 table with an associated χ^2 statistic calculated in the standard fashion. The χ^2 statistic associated with the observed aggregation of cases and mutations was compared with those calculated from 2,000 random permutations of families and mutations. The rank of the observed χ^2 statistic among those from 2,000 replicates is the nominal P for testing the association between the prevalence of ovarian cancer and a specific mutation. The S-Plus package (StatSci) was used to perform the randomization test. Phenotypic analysis of age at diagnosis, among mutations, was performed by the T-TEST, GLM, and VARCOMP procedures of the SAS statistical analysis package.

Results

Haplotype Analysis and Age of Mutations

The mutations described in this report span the *BRCA2* gene and are small insertions or deletions that cause protein truncation. In table 2, the mutations are characterized as to the number of families studied, the numbers of genotypes and haplotypes obtained, and the geographic diversity (as based on the number of countries from which samples were contributed). The most common haplotype associated with each of the nine mutations studied, as well as the estimated G, support interval, and estimated heterogeneity for those mutations with at least five haplotypes to analyze are also shown in table 2. Although the estimation of the ages of the mutations incorporated data from all 10 markers, we report the consensus haplotype at the six markers closest to *BRCA2*, since, in many cases, the haplotype beyond these markers was difficult to determine. For four of the five mutations examined, the estimated fraction of families in which cancer was due to an independent mutational event was 0; for 6503delTT, the estimated proportion was .11, which is not significantly different from 0. For 6174delT, the 1-LOD upper bound for the proportion attributable to one (or more) independent identical mutations was .06. In all cases, there was no significant evidence of mutational heterogeneity, indicating that, for each mutation studied, all families with the mutation represent derivations from a single ancestral haplotype on which the mutation arose. The estimates of G are based on assumptions about mutation rates and recombination rates and therefore may be more appropriately considered as relative indications of time since the mutation originated, rather than as absolute values. We estimate the 982del4, as an example, to have occurred relatively recently—that is, 18 generations ago (1-LOD support interval 4–43), or ~360 years ago (1-LOD support interval 80-860 years).

Association between Phenotypic Variation and Mutations

A summary of the number of cases of breast and ovarian cancers and the ages at diagnosis of the breast cancer cases, stratified by *BRCA2* mutation type, is shown in table 3, for all families with all breast cancer cases and for those "high-risk" families (as described in the Subjects and Methods section) that have breast cancer cases diagnosed at age <60 years. There was significant variation in age at diagnosis among the nine mutations tested when all cases in all families were considered ($P = .0007$, by nested ANOVA), as well as when the analysis was restricted to high-risk families and cases diagnosed at age <60 years ($P = .015$), although only ~8% and ~6%, respectively, of the variance was explained by individual mutation. In both analyses, there was no evidence of significant variation between families, for any mutation group, and the variance due to this effect was estimated to be zero in both cases.

Table 3

Summary of Phenotypic Data Associated with Mutation

	All Families[a]				Families with ≥3 Cases[b]			
		No. of Cancer Cases				No. of Cancer Cases		
Mutation	No.	Female Breast (Age [years])	Ovarian	Male Breast	No.	Female Breast at Age <60 Years (Age [years])	Ovarian	Male Breast
982del4	5	25 (41)	1	4	4	20 (38)	1	4
2041insA	5	16 (41)	4	5	4	11 (39)	3	5
3034del4	11	37 (42)	6	2	9	33 (42)	5	2
4486delG	5	16 (48)	0	3	1	6 (44)	0	0
5573insA	3	5 (47)	7	0	2	2 (40)	7	0
6174delT[c]	67	119 (49)	29	12	22	60 (46)	12	8
6503delTT	7	20 (44)	12	1	6	18 (44)	12	1
9254del5	3	16 (48)	3	3	3	11 (43)	3	3
9326insA	4	9 (34)	0	2	1	3 (35)	0	1
Total	110	263 (45.6)	62	32	52	164 (42.7)	43	24

[a] Includes all families on which at least some phenotypic information was available. Breast cancer tabulation contains all cases of breast cancer, regardless of age, as well as those cases for which age at diagnosis is unknown.

[b] Families with at least three cases of cancer, where a case is defined as a female breast cancer at age <60 years, an ovarian cancer, or a male breast cancer. Only the cases of female breast cancer at age <60 years are included in the results.

[c] In 13 families obtained from a consecutive series of Ashkenazi Jewish ovarian cancer patients tested only for the 6174delT mutation, the ovarian cancer proband was omitted from this table and subsequent analyses; however, the proband was used in determining whether the family had three or more cases.

Examination of the OCCR

The randomization test described in the Subjects and Methods section was used to examine possible differences in the relative proportions of cases of breast and ovarian cancers, for mutations inside and outside the OCCR. These results are shown in table 4. It is clear that there is a higher proportion of ovarian cancer cases associated with families with mutations in the OCCR region, although this difference is not significant for either the complete data set ($P = .12$) or the high-risk subset ($P = .11$). The odds ratio for the entire set of families is 2.1. Interestingly, when we examined the age at diagnosis of the breast cancer cases in terms of OCCR status, we found that most of the age-at-onset variation between mutations could be ascribed to the location relative to the OCCR. This difference, of older age at onset for the OCCR region, was highly significant, both for the nested analysis of variance with between-family variation used as the error term and by ordinary *t*-test. Because the 6174delT mutation group was the largest and had the oldest age at onset, we also performed the analysis of age at onset and OCCR again, without this group. When we removed the cases with a 6174delT mutation, the effect of the mutation location in the OCCR is still present but is not significant ($P = .09$).

Discussion

In this paper, we have analyzed genotypic and phenotypic data from a series of breast cancer families and from isolated cases with one of nine recurrent mutations in the *BRCA2* gene. These data appear to include both population-specific sequence variants, as well as those found in more geographically diverse populations of northern European Caucasian ancestry. The mutation with the oldest estimated age, 3034del4, was found in the most diverse set of samples (except for the 6174delT mutation in the Ashkenazi population), both in multiple centers in the same country and in seven different countries. For the mutations studied, the multiple instances of specific mutations generally appear to represent founder effects many generations in the past, rather than independent mutational events. This is in contrast to the *BRCA1* mutations—4184del4, Arg1443ter, and 185delAG—which, on the basis of the multiple origins of these mutations, may represent hot spots (Neuhausen et al. 1996*b*).

The 4486delG mutation has been reported only in Scandanavia (Håkansson et al. 1997). For this study, there were too few haplotypes to determine the age of the mutation. However, all four samples (three from sporadic male breast cancer cases and one large breast cancer family) genotyped with this mutation appeared to share a conserved haplotype over an ~3-cM interval containing the *BRCA2* locus. A similar pattern was observed in the three Dutch families carrying the 5573insA mutation. The 9254del5 mutation has been identified only in two French families of Catalan origin and in a single Spanish family also from this region. The three families share a conserved haplotype over an ~2-cM region spanning the *BRCA2* locus. These three families have different phenotypes, with one family having three cases

Am. J. Hum. Genet. 62:1381–1388, 1998

Table 4

Examination of OCCR

Mutation Location	All Families[a]				Families with ≥3 Cancer Cases[b]			
		No. of Cancer Cases				No. of Cancer Cases		
	No.	Female Breast Age (years)	Ovarian	Male Breast	No.	Female Breast at Age <60 Years (Age [years])	Ovarian	Male Breast
OCCR$^-$	82	160 (48.0)	48	17	31	88 (44.9)	31	9
OCCR$^-$	28	103 (41.9)	14	16	21	76 (40.3)	12	15

[a] As defined in table 3. For age at diagnosis, $P < .0001$; for breast cancer versus ovarian cancer, $P = .12$.

[b] As defined in table 3. For age at diagnosis, $P < .0005$; for breast cancer versus ovarian cancer, $P = .11$.

of male breast cancer and four cases of female breast cancer, a second family having three cases of ovarian cancer, and a third family having eight site-specific cases of female breast cancer.

By contrast, the 3034del4 mutation has been found in families in seven different western European and North American countries (Belgium, Canada, France, Italy, Spain, Switzerland, and the United States). There was a considerable amount of haplotype diversity among the 11 families examined, accounting for the large value of the estimated age. Although our analysis failed to find significant statistical evidence of multiple independent origins for this mutation (the maximum-likelihood estimate for the proportion due to independent mutation is 0), given the limited number of families available for analysis, statistically we could not rule out the possibility that there were independent mutations for as many as half the families. This mutation is in a region that may be a hot spot for such deletions. Another 4-bp deletion, located only 2 bp downstream, has been reported in five families thus far, and a 2-bp deletion located 4 bp downstream has been reported once (Breast Cancer Information Core).

Of particular interest is the 6174delT mutation found in high frequency in the Ashkenazi Jewish population. Along with the two *BRCA1* mutations (185delAG and 5382insC), it has been estimated that 1 in 50 Ashkenazi Jewish individuals carry one of these three mutations (Struewing et al. 1995, 1997; Oddoux et al. 1996; Roa et al. 1996). These mutations account for ~30% of early-onset breast cancer (Neuhausen et al. 1996*a*; Offit et al. 1996; Tonin et al. 1996) and for as much as 60% of all ovarian cancer in this population (Abeliovich et al. 1997). On the basis of our analysis of haplotypes and genotypes of 69 families with the 6174delT mutation, we estimate that the mutation originated ~29 generations ago (1-LOD support interval 22–38). The corresponding analysis for the age of the *BRCA1* 185delAG, on the basis of our original set of 18 families with this mutation, resulted in an estimate of 46 generations (1-LOD support interval 22–82) and suggested that the cases in ~90% of the families are due to the presumed ancestral Jewish mutation (an estimate reflecting the fact that two families of non-Jewish ancestry were part of the sample). Thus, the 6174delT mutation appears to have originated more recently. Support for the more recent origin of the 6174delT mutation comes from examination of these mutations in 44 non-Ashkenazi Jewish patients. One Iraqi patient had a 185delAG mutation, and none had a 6174delT mutation (Abeliovich et al. 1997). Sher et al. (1996) also reported a 185delAG mutation in an Iraqi Jew, suggesting that this mutation has an origin earlier than that of the 6174delT mutation. More recently, an additional three *BRCA1* 185delAG mutations have been identified, in a sample of 639 Iraqi Jews (Bar-Sade et al. 1997), but, to our knowledge, the 6174delT mutation has never been found outside the Ashkenazi Jewish population.

Our analysis was consistent with the finding by Gayther et al. (1997)—that is, that there is a higher incidence of ovarian cancer relative to breast cancer associated with the OCCR; however, this higher incidence was not statistically significant. One possible reason for the difference between the significance presented here and that reported by Gayther et al. (1997) could be the ill-defined 5′ end of the OCCR. The 3034del4 mutation is on the 5′ border of the OCCR, as defined by Gayther et al. (1997), and its exclusion, rather than inclusion in the OCCR, could have an affect on the analysis.

Among the mutations, there were significant differences associated with age at diagnosis of breast cancer. Much of the variation was associated with mutation location relative to the OCCR. However, when we removed the cases with a 6174delT mutation, the effect of the mutation location in the OCCR, although still present, was not significant. The later age at onset of breast cancer in the cases with the 6174delT mutation could be due to ease of screening families for this common mutation. However, the age effect is still present in those families with three or more cancer cases who would likely be screened in any testing program, suggesting that mutations within the OCCR and/or, more specifically, the 6174delT mutation do confer a later age at onset of breast cancer. On the basis of previous studies

of two common mutations, there is a suggestion that mutations in the OCCR are less penetrant for breast cancer at a younger age. In the Icelandic studies of the 999del5 mutation, which is outside the OCCR, 28% of Icelandic breast cancer cases of age <40 years carry this mutation, which has a population prevalence of 0.50%. In contrast, for the 6174delT mutation, which is within the OCCR, 8% of Ashkenazi Jewish breast cancer cases of age <40 years carry this mutation, which has a population prevalence of 1.2%. Therefore, with a prevalence twofold higher for the 6174delT mutation, there is a large difference, in comparison with the Icelandic mutation, for age at onset of breast cancer, suggesting lower penetrance at age <40 years.

As a first step in mutation detection, comparison of an observed haplotype in a family examination of haplotypes can be useful to identify common mutations. In addition to this set of haplotypes for recurrent mutations, we are also constructing a haplotype database of any mutations, so that others can compare their haplotypes (for further information, please contact S.L.N.). A haplotype database of Dutch mutations is available from a Leiden University Medical Center Department of Human Genetics Website. Since multiple families with identical mutations on identical genetic backgrounds can be ascertained, this will allow us to better elucidate additional genetic and environmental factors that contribute to the observed variation in phenotype. Similarly, studies of families with identical mutations but with different origins will allow us to examine better the possible effect of genetic modifier loci. A copy of the revised version of the haplotype-analysis program is available, on request, from D.E.G.

Acknowledgments

The authors wish to thank the many families who participated in the research studies described in this article. This work was supported by U.S. Department of Defense grants DAMD-17-94-J-4260 and DAMD 17-96-I-6266 (both to S.L.N.), by the Canadian Breast Cancer Foundation and U.S. Department of Defense grant DAMD-17-J-4299 (both to S.A.N.), by National Institutes of Health grant HG-00571 (to D.G.), by Hungarian research grants OMFBIEU 96-09-004 and OTKA T19307 (both to E.O.), by Spanish Ministry of Education grant CICYT 96/0192 (to A.O. and J.B.), by National Institutes of Health grant R01 CA27632 (to E.S. and M.-C.K.), by Swedish Cancer Society grants (to A.B.), by an Italian Association for Cancer Research grant (to M.A.C.), and by Cancer Research Campaign program grants. B.A.J.P. is a Gibb Fellow of the CRC. This work was also supported by the Utah Cancer Registry, which is funded by National Cancer Institute contract N01-CN-6700, with additional support from the Utah Department of Health and the University of Utah. We also want to thank clinicians and researchers at all the institutions, including Håkan Olsson, Oskar Johannsson, Niklas Loman, and Ulf Kristoffersson from the Oncogenetic Group at University Hospital in Sweden; and Mary Daly, Josephine Costalas, and JoEllen Dangel from Fox Chase Cancer Center. We also thank Thao Tran for performing the genotyping at the University of Utah. A.O. is a fellow of the Funación Conchita Rábago.

Electronic-Database Information

URLs for data in this article are as follows:

Breast Cancer Information Core, http://www.nchgr.hih.gov/intramural_research/Lab_transfer/Bic

Leiden University Medical Center Department of Human Genetics ("Haplotypes carrying *BRCA1* mutations found repeatedly in the Dutch population"), http://ruly70.medfac.leidenuniv.nl/~devilee/hapover.htm

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The *APC* I1307K allele and breast cancer risk

The I1307K variant of *APC* occurs in approximately 6% of Ashkenazi Jews and has been associated with familial colorectal neoplasia. In four of the eight pedigrees characterized by Laken *et al.*[1], women who may be heterozygous for this allele were diagnosed with breast cancer. Animal models suggest a relationship between *Min*, a mutant allele of the mouse *Apc* gene, and breast neoplasia, with approximately 5% of B6 *Min*/+ females reported to develop breast tumours[2]. Loss of heterozygosity or comparative genomic hybridization studies have identified 5q or the *APC* region as sites of loss in both *BRCA1*-mutated and *BRCA1* wild-type breast tumours[3–6], suggesting a possible role for *APC* in the progression of a subset of breast tumours. We speculated that Ashkenazi Jewish women with breast cancer may be more likely to have the I1307K allele than unaffected Ashkenazi Jewish controls.

We determined the frequency of this polymorphism in 632 unrelated women with primary invasive breast cancer who were not selected for a family history of breast or ovarian cancer, and who self-reported as being of Ashkenazi Jewish descent. Cases were diagnosed at three centres: 216 in Toronto, 215 in New York (Memorial Sloan-Kettering Cancer Center (MSKCC)) and 201 in Montreal (SMBD-Jewish General Hospital). The Montreal cases were all diagnosed before age 65, whereas 73 of the 215 MSKCC cases were diagnosed before age 42. The remainder of the 632 cases were unselected for age. For 115 of the MSKCC cases, and all Montreal cases, the field for religion on the medical chart was used to select self-identified individuals of Jewish religion with breast tumour tissue available for study. Paraffin blocks were obtained and analysed anonymously. In 100 cases at MSKCC and all cases at Toronto, questionnaires were used to determine religious and geographical background from individuals with personal histories of breast cancer, irrespective of whether they had a family history of the disease. DNA was obtained from peripheral lymphocytes for these samples.

We studied familial breast cancer cases by recruiting affected Ashkenazi Jewish individuals with at least two first or second degree relatives with breast and/or ovarian cancer from the Fox Chase Cancer Center (n=74), the University of Pennsylvania Medical Center (n=59), MSKCC (n=53) and the Dana-Farber Cancer Center (n=36). The prevalence of the I1307K allele was estimated in a control group of 4,635 unrelated individuals of self-reported Ashkenazi Jewish ancestry who were participating in a Washington D.C. area genetic epidemiologic study[7] and in 320 volunteers for Tay-Sachs screening in California. IRB approval of the current study was granted at all participating institutions.

Although many techniques (allele specific hybridization, single-strand conformation analysis, conformation-sensitive gel electrophoresis and amplification-created restriction site; refs 8–10) were used to detect the I1307K polymor-phism, all positive cases were confirmed by direct sequencing of PCR products. All cases were also analysed for the 185delAG, 5382insC (*BRCA1*) and 6174delT (*BRCA2*) founder mutations. The hypothesis of no disease-mutation exposure was tested using a chi-square test for association; odds ratios, as estimates of the relative risk of breast cancer in association with the I1307K allele, were calculated with 95% confidence intervals.

We first analysed the family history-unselected cases. Sixty-six of 632 breast cancer cases were heterozygous for the I1307K allele (10.4%; Table 1). This proportion was significantly greater than the 7.03% carrier frequency observed in Ashkenazi Jewish volunteers from the Washington D.C. study[7] ($P=0.003$), corresponding to an odds ratio (OR) of 1.5 (95% CI = 1.2–2.0). This estimate of the carrier frequency in Ashkenazi Jewish controls is probably high, because the frequency in the Washington study cohort was 6.8% when individuals with self-reported histories of cancer were excluded from analysis[7]. We also observed a control frequency of 5.6% for the allele in 320 volunteers for Tay-Sachs testing. When the group selected for family history were included in the analysis, the magnitude of the effect decreased (OR = 1.4, 95% CI = 1.1–1.8), but only 17 individuals from high-risk clinics carried the I1307K allele. Moreover, restricting the analysis to *BRCA* mutation carriers revealed a possible association between the three *BRCA* founder mutations and *APC* polymorphism status; the frequency of the I1307K allele in the subset of patients with *BRCA* mutations was significantly greater than that observed in the control population (OR = 1.9, 95% CI = 1.2–3.0).

These data, and the similar frequencies of the *APC* polymorphism in patients without *BRCA* mutations compared with the control population (OR = 1.4, 95% CI = 1.0–1.8; Table 1), suggest that the effect of the I1307K allele on breast cancer risk is largely or entirely limited to those with *BRCA* founder mutations. This effect does not appear to operate through a shift in age-dependent penetrance, as double heterozygotes for *BRCA* founder mutations and the I1307K allele had the same mean age as women heterozygous only for *BRCA* founder mutations. In addition, in the combined group of 731 cases with a known age at diagnosis, as well as in subgroups sorted by age or family history, there was no significant difference be-

Table 1 • Frequency of I1307K in populations studied

Group	Subgroup	I1307K carriers	Total	%	OR	95% CI	*P*
Controls[a]		326[b]	4635	7.0	1.0	—	—
Unselected cases							
	BRCA1/+	11	65	16.9	2.7	1.4–5.2	0.005
	BRCA2/+	5	26	19.2	3.1	1.2–8.4	0.04
	BRCA/+	16	91	17.6	2.6	1.5–4.6	0.001
	+/+ (wt)	50	541	9.2	1.4	1.0–1.8	0.07
	All	66	632	10.4	1.5	1.2–2.0	0.003
High risk clinic cases[c]							
	BRCA1/+	8	81	9.9	1.5	0.7–3.0	0.4
	BRCA2/+	0	17	0.0	—	—	—
	BRCA/+	8	98	8.2	1.2	0.6–2.4	0.8
	+/+ (wt)	9	124	7.3	1.0	0.5–2.1	0.9
	All	17	222	7.7	1.1	0.7–1.8	0.8

BRCA1/+ signifies heterozygotes for either the 185delAG, 5382insC *BRCA1* and 6174delT *BRCA2* mutations; +/+(wt) signifies wild type (for *BRCA1/2*). [a]Population controls from Woodage *et al.*[7] [b]Two controls were homozygous for the I1307K allele; percentage is carrier frequency from series by Woodage *et al.*[7] [c]MSKCC, Fox Chase Cancer Center, the Cancer Risk Evaluation Program at the University of Pennsylvania and the Dana-Farber Cancer Center.

tween age at onset of breast cancer in I1307K heterozygotes compared to *APC* wild-type patients. These findings are consistent with possible interactions of the I1307K *APC* polymorphism with other as-yet-unidentified modifying factors, including both high- and low-penetrance alleles associated with familial cancer risk. Such modifiers, if over-represented in cases selected for extensive family history of disease, could account for the lower observed prevalence of the *APC* variant allele in this group.

A previous study did not find an excess of the I1307K polymorphic allele in 158 Ashkenazi Jewish kindreds with breast or colorectal cancers, but was not able to exclude co-segregation of the polymorphism with breast cancers in 9 of 10 pedigrees shown[11]. When combined with the results reported by Woodage *et al.* in this issue[7], the I1307K polymorphism emerges as a candidate low-penetrance breast cancer susceptibility allele or a genetic modifier of risk in *BRCA* heterozygotes. In the current study, there was no significant association between breast cancer risk and the *APC* polymorphism in patients without germline *BRCA* mutations. If we assume that the effect of the I1307K allele is restricted to *BRCA* mutation carriers (approximately 2.5% of the Ashkenazi Jewish population), then 6% of breast cancers among *BRCA* heterozygotes are attributable to the I1307K allele. Therefore, although of genetic epidemiologic interest, these findings indicate that for the substantial majority of individuals of Ashkenazi Jewish background, clinical testing for the I1307K allele is not justified outside of a research context.

Acknowledgements

We are grateful to L. Norton, J. Garber, C. Lerman, L. Petrukhin, S. Narod, H. Ostrer and T. Rebbeck. K.N. is supported by NIH training grant 1TG32GM08638. B.W. is supported by NIH RO1 CA5760 and the Breast Cancer Research Foundation. S.L.N. is supported by NIH RO1-CA7415 and DAMD-17-94-J4260. Study funding: National Institutes of Health (CA63720), the Society of Memorial Sloan-Kettering, the Breast Cancer Research Foundation, the Lymphoma Foundation, Fonds de la recherche en sante du Quebec, the Judy Steinberg Research Fund, the National Cancer Institute of Canada, the Canadian Cancer Society and the Canadian Breast Cancer Research Initiative, as well as resources made available by the Cooperative Family Registry for Breast Cancer Studies, supported by NIH UO1 CA69467-01.

Mark Redston[1,2], Katherine L. Nathanson[7], Zhi Qiang Yuan[6], Susan L. Neuhausen[9], Jaya Satagopan[5], Nora Wong[6], Diana Yang[5], Doudja Nafa[5], John Abrahamson[4], Hilmi Ozcelik[1,2], Danielle Antin-Ozerkis[7], Irene Andrulis[1,2], Mary Daly[8], Leonard Pinsky[6], Deborah Schrag[10], Steven Gallinger[1,2], Michael Kaback[11], Mary-Claire King[12], Trevor Woodage[13], Lawrence C. Brody[13], Andrew Godwin[8], Ellen Warner[2,3], Barbara Weber[7], William Foulkes[6] & Kenneth Offit[5]

[1]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, [2]Ontario Cancer Genetics Network, [3]Sunnybrook Regional Cancer Centre, [4]Centre for Research in Women's Health, University of Toronto, Ontario, Canada. [5]Departments of Human Genetics, Biostatistics and Epidemiology, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. [6]Cancer Prevention Research Unit, SMBD-JGH, Departments of Medicine, Oncology, Surgery, Human Genetics and Montreal General Hospital Research Institute, McGill University, Montreal, Quebec, Canada. [7]Departments of Medicine and Genetics, Stellar-Chance Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania, USA. [8]Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA. [9]Genetic Epidemiology Group, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah, USA. [10]Dana Farber Cancer Center, Boston, Massachusetts, USA. [11]Children's Hospital, University of California at San Diego, California, USA. [12]Department of Genetics, University of Washington, Seattle, Washington, USA. [13]Genetics and Molecular Biology Branch, NHGRI, NIH, Bethesda, Maryland, USA. M.R., K.C.N., Z.Q.Y. and S.L.N. contributed equally to this work. Correspondence should be addressed to W.F. (e-mail: MDWF@musica.mcgill.ca).

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Cancer Risks in BRCA2 Mutation Carriers

The Breast Cancer Linkage Consortium

Background: Carriers of germline mutations in the BRCA2 gene are known to be at high risk of breast and ovarian cancers, but the risks of other cancers in mutation carriers are uncertain. We investigated these risks in 173 breast–ovarian cancer families with BRCA2 mutations identified at 20 centers in Europe and North America. *Methods:* Other cancer occurrence was determined in a final cohort of 3728 individuals, among whom 681 persons had breast or ovarian cancer and 3047 persons either were known mutation carriers, were first-degree relatives of known mutation carriers, or were first-degree relatives of breast or ovarian cancer patients. Incidence rates were compared with population-specific incidence rates, and relative risks (RRs) to carriers, together with 95% confidence intervals (CIs), were estimated by use of a maximum likelihood approach. Three hundred thirty-three other cancers occurred in this cohort. *Results:* Statistically significant increases in risks were observed for prostate cancer (estimated RR = 4.65; 95% CI = 3.48–6.22), pancreatic cancer (RR = 3.51; 95% CI = 1.87–6.58), gallbladder and bile duct cancer (RR = 4.97; 95% CI = 1.50–16.52), stomach cancer (RR = 2.59; 95% CI = 1.46–4.61), and malignant melanoma (RR = 2.58; 95% CI = 1.28–5.17). The RR for prostate cancer for men below the age of 65 years was 7.33 (95% CI = 4.66–11.52). Among women who had already developed breast cancer, the cumulative risks of a second, contralateral breast cancer and of ovarian cancer by the age of 70 years were estimated to be 52.3% (95% CI = 41.7%–61.0%) and 15.9% (95% CI = 8.8%–22.5%), respectively. *Conclusions:* In addition to the large risks of breast and ovarian cancers, BRCA2 mutations may be associated with increased risks of several other cancers. [J Natl Cancer Inst 1999;91: 1310–6]

The majority of families with a clearly dominant predisposition to breast and/or ovarian cancer are now known to harbor germline mutations in either BRCA1 or BRCA2 genes *(1–3)*. More than 100 distinct disease-causing mutations in BRCA2 have been found since its identification in 1995. BRCA2 mutations are known to predispose individuals to a high lifetime risk of breast cancer, similar to that associated with BRCA1 mutations, together with a lower, although still statistically significant, risk of ovarian cancer *(3)*.

In addition to the risks of breast and ovarian cancers, several reports have suggested that BRCA2 mutations may be associated with an increased risk of other cancers. Easton et al. *(4)* studied two of the largest known families linked to BRCA2, from Utah in the United States and from Ireland, respectively. They found a statistically significant excess of prostate cancer, with a relative risk (RR) of 2.69 based on five possible carriers, and of laryngeal cancer, with an RR of 7.67 based on two possible mutation carriers. They also found one confirmed and one possible case of ocular melanoma in obligate carriers. Further support for the prostate cancer risk was provided by Struewing et al. *(5)* in their study of BRCA1 and BRCA2 mutations in Ashkenazi Jewish volunteers from the Washington, DC, area. On the basis of the family histories of known mutation carriers, they estimated a cumulative risk of prostate cancer of 16% by the age of 70 years, with no statistically significant difference between BRCA1 and BRCA2 carriers. An excess risk of prostate cancer has also been reported in relatives of breast cancer patients from Iceland *(6)* and specifically in multiple-case breast cancer families, the majority of which are due to a single founder BRCA2 mutation 999del5 *(7,8)*. Johannesdottir et al. *(9)* found the BRCA2 mutation 999del5 in two of 75 prostate cancer case patients diagnosed below the age of 65 years, compared with two of 499 in Icelandic population control subjects. An association between BRCA2 and pancreatic cancer has also been suspected, since homozygous deletion of BRCA2 in a pancreatic adenocarcinoma has been observed *(10)*. Several pancreatic cancers have been observed in BRCA2 families [e.g., *(11)*]. In addition, Goggins et al. *(12)* found probable germline BRCA2 mutations in three of 15 pancreatic cancer patients with loss of heterozygosity (LOH) at the BRCA2 locus in the tumor and two further mutations in a limited screen of 245 unselected patients with pancreatic cancer. These proportions are higher than likely population frequencies, but the magnitude of the excess is hard to evaluate, particularly since three of the mutations were the 6174delT mutation, which is highly prevalent in Ashkenazi Jews. Katagiri et al. *(13)* found no mutations among 36 Japanese patients with pancreatic cancer.

To provide a more comprehensive assessment of the cancer risks to BRCA2 mutation carriers, we have studied the risks of cancer in the large series of families collected by the Breast Cancer Linkage Consortium (BCLC). To our knowledge, this is by far the largest series of BRCA2 families and carriers currently available. We have also used data on the occurrence of bilateral breast cancer and ovarian cancer subsequent to breast cancer to provide further estimates of the risks of breast and ovarian cancers in mutation carriers.

Subjects and Methods

Families

Families were ascertained from 20 centers in Western Europe, the United States, and Canada that were studying familial breast or ovarian cancer. Thirteen families from the Toronto group were families of Ashkenazi Jewish patients from North American centers with ovarian cancer who tested positive for the 6174delT mutation. Eight of the Swedish

Affiliation of authors: Cancer Research Campaign (CRC) Genetic Epidemiology Unit, Strangeways Research Laboratory, Cambridge, U.K.

Correspondence to: Douglas Easton, Ph.D., CRC Genetic Epidemiology Unit, Strangeways Research Laboratory, Worts Causeway, Cambridge, U.K. (e-mail: douglas@srl.cam.ac.uk).

See "Appendix" section for a list of the consortium members.

See "Notes" following "References."

families were ascertained through mutation testing of an unselected series of male breast cancer patients (six families) or ovarian cancer patients (two families), and two additional families were ascertained through testing of breast cancer cases among women under the age of 30 years. All of the remaining families were ascertained on the basis of at least two relatives with breast cancer diagnosed below age 60 years or ovarian cancer diagnosed at any age, with more restrictive criteria in some centers. There were 173 families, ranging in size from three to 255 individuals, with the median family size being 17. These families contained 596 female breast cancer patients diagnosed under the age of 60 years, 132 ovarian cancer patients, and 72 male breast cancer patients. Eighty-eight of the female breast cancer patients and four of the male breast cancer patients had bilateral disease. Thirty-nine families contained five or more female breast cancer patients under 60 years of age, 76 contained at least one ovarian cancer patient, and 53 contained at least one male breast cancer patient.

Families were eligible for this study if one or more affected individuals tested positive for a pathologic BRCA2 mutation or if there was clear evidence of linkage to BRCA2, with LOD (i.e., logarithm of the odds) scores greater than 1.0. In practice, only four families were included on the basis of linkage alone. A total of 97 distinct mutations (70 frameshift, 15 nonsense, six missense, and six splice site) were observed in 169 families; one family had both a frameshift and a missense mutation. The mutations accounting for the larger number of families were 6174delT (23 families), 999del5 (14 families), 8764delAG (11 families), 3034del4 (seven families), 6503delTT (six families), and 4486delG (six families). All other mutations occurred in three or fewer families.

For all available families, basic follow-up information, including dates of birth, death, and last observation and dates and types of all cancers, was requested for all patients with breast or ovarian cancer and all of their first-degree relatives and from all known carriers and all of their first-degree relatives. Information on mastectomies and oophorectomies was also recorded. We also requested information on carrier status, when known, as determined by either direct mutation testing or segregation of linked haplotypes. All cancers were coded according to the 9th revision of the International Classification of Diseases (ICD) *(14)*. A total of 566 cancers, excluding breast cancer, ovarian cancer, and nonmelanoma skin cancer, were reported among the 4778 individuals who were either affected with breast or ovarian cancer, known mutation carriers, or first-degree relatives of these individuals. Of these, 269 (48%) were confirmed by pathology report, clinical records, or death certificate. All participating centers of the study had received approvals from the respective ethical committees of their institutions.

Statistical Methods

The principal aim of this study was to estimate the risks of cancer in mutation carriers. We first constructed a cohort of the following individuals: *(a)* women affected with breast cancer under the age of 60 years or with ovarian cancer at any age or men affected with breast cancer at any age (800 individuals, 363 of whom were known mutation carriers), *(b)* unaffected known mutation carriers (622 individuals), or *(c)* first-degree relatives of affected individuals in category *a* or of known carriers (3271 individuals). For these purposes, a woman diagnosed with her first breast cancer at the age of 60 years or older and who had not had an ovarian cancer was included in the "unaffected" category. Eighty-five breast or ovarian cancer patients were shown to be noncarriers of the disease causing mutation in that family ("sporadic" cases) and were ignored in this analysis, leaving 4693 individuals to be considered further.

To compute incidence rates for individuals not affected with breast or ovarian cancer, follow-up was deemed to commence on their date of birth or on January 1, 1960, whichever was the later, and to cease on the date of their first cancer, their date of death or loss to follow-up, their 85th birthday, or on December 31, 1995, whichever occurred first. Follow-up before 1960 was ignored to minimize errors in classification of tumors and because reliable population-specific incidence rates were available for almost all centers from that date, but often not before. We also excluded all individuals born before January 1, 1890. Follow-up for individuals affected with breast or ovarian cancer was similar, except that it commenced on the date of their first cancer rather than on the date of their birth and ceased on the date of their second cancer.

Since ascertainment was based on a minimum number of breast or ovarian cancer cases within a family, to include these ascertainment-influencing events in the analysis would bias the results. We were unable to determine reliably the specific cases in each family responsible for that family's ascertainment; therefore, to correct for this ascertainment bias, it was necessary to ignore all follow-up prior to and including the first breast or ovarian cancer for every individual.

After those cases with no follow-up in the relevant period were removed, the final cohort comprised 3728 individuals, of whom 50 were men with breast cancer and 631 were women with breast cancer below the age of 60 years or with ovarian cancer at any age. Among the unaffected individuals, there were 471 known carriers, 390 known noncarriers, and 2186 persons of unknown carrier status. One hundred forty-eight of the 471 unaffected identified carriers were "obligate carriers" (i.e., individuals known to be carriers by virtue of their position in the family, such that a mutation in a descendant must have been inherited through them). The remaining 323 carriers were identified by direct testing. Of the 1050 total individuals excluded, 236 were lost to follow-up. Other than breast or ovarian cancer, 333 cancers occurred in the cohort, of which 176 (53%) were confirmed.

Expected numbers of cancers were computed in the usual manner by multiplying person-years at risk by the appropriate age-, sex-, period-, site-, and population-specific incidence rates, by use of the program Person-Years *(15)*. The relevant rates were obtained from the publications "Cancer Incidence in Five Continents" *(16–20)* together with information provided by the International Agency for Research on Cancer. The calendar periods into which rates were divided differed slightly between registries, according to the data available, but most periods covered 5 years. For the U.S. centers, we used rates from the Surveillance, Epidemiology, and End Results (SEER) Program[1] for the periods from 1973 onward. Rates for whites were used, since almost all of the families were from this group. SEER rates were not available before 1973, and we used instead the rates for Alameda, CA. For Montreal, we used Quebec rates; for all of the other centers, we used country-specific rates.

To provide unbiased estimates of the RR for each cancer, it was necessary to combine data on the observed risks to known carriers and those relatives whose carrier status was unknown. (Estimates based only on typed carriers would have been biased, since the probability of being typed could be influenced by disease status.)

As a first approximation to the RR ($\hat{\lambda}$) for each site, we used the following formula:

$$\hat{\lambda} = \frac{\sum w_i O_i}{\sum w_i E_i}, \qquad [1]$$

where O_i is 1 if individual i is affected and 0 otherwise, and E_i is the number of cancers expected for individual i under the null hypothesis of no BRCA2-associated risk, i.e., on the basis of population rates as described above. w_i is the probability that individual i is a mutation carrier, given his or her phenotypic status (age, sex, and disease status) and position in the pedigree. The probabilities w_i were computed by use of a standard procedure for computing genetic risks, with the use of the program MENDEL *(21)*. In these computations, we assumed that the risks of breast and ovarian cancers in BRCA2 mutation carriers were those estimated in the previous analysis of BCLC families *(3)*.

We also estimated RRs ($\hat{\phi}$) for noncarriers using the following analogous formula:

$$\hat{\phi} = \frac{\sum (1 - w_i) O_i}{\sum (1 - w_i) E_i}. \qquad [2]$$

Since the distributions of these estimated RRs were complex mixtures of Poisson distributions, we constructed statistical tests of the hypotheses that the RRs were greater than 1 by simulation. We derived the distribution of the estimated risk under the null hypothesis of no excess risk by simulating each O_i 10 000 times as a random draw from a Poisson distribution with mean E_i. Significance levels were then computed as the proportion of simulated datasets for which the RR exceeded the observed value multiplied by 2 to give two-sided P values.

The above procedure provides significance tests for the RR but does not provide consistent estimates of the RRs because the carrier probabilities w_i do not take into account the phenotypic status with regard to the site of interest. We, therefore, computed maximum likelihood estimates of λ and ϕ using the EM algorithm *(22)*. In this procedure, the carrier probabilities w_i, given the initial estimates of λ and ϕ, were re-estimated with the use of the Bayes formula:

$$w_i' = \frac{w_i \lambda^{O_i} \exp(-\lambda E_i)}{w_i \lambda^{O_i} \exp(-\lambda E_i) + (1 - w_i) \phi^{O_i} \exp(-\phi E_i)} = \left[1 + \left(\frac{1}{w_i} - 1 \right) \left(\frac{\phi}{\lambda} \right)^{O_i} \exp(E_i(\lambda - \phi)) \right]^{-1}. \qquad [3]$$

Iterative re-estimation of the carrier probabilities and RRs with the use of formulae 1–3 leads to maximum likelihood estimates in the usual way. Confidence intervals (CIs) for λ and ϕ were derived from standard estimates for the variance–covariance matrix for estimates obtained with the use of the EM algorithm *(22)*, which reduces in this case to:

$$Var(\log\hat{\lambda},\log\hat{\phi}) = \begin{bmatrix} \sum w_i O_i - \sum w_i(1-w_i)(O_i - \hat{\lambda}E_i)^2 & \sum w_i(1-w_i)(O_i - \hat{\lambda}E_i)(O_i - \hat{\phi}E_i) \\ \sum w_i(1-w_i)(O_i - \hat{\lambda}E_i)(O_i - \hat{\phi}E_i) & \sum (1-w_i)O_i - \sum w_i(1-w_i)(O_i - \hat{\phi}E_i)^2 \end{bmatrix}^{-1}. \quad [4]$$

In practice, joint estimation of λ and ϕ led, in most cases, to estimates of the RR ϕ to noncarriers that were not significantly different from 1, and the RR for all sites combined was very close to 1. To simplify the analyses (and to gain some precision), the estimates of λ presented in the tables have been derived under the restriction that $\phi = 1$, and we have commented explicitly where this assumption may not be justified. Statistical tests of the hypotheses that the RR estimates differed by age or by center were constructed in the usual way from the above variance estimates. These tests were two-sided.

The risks of breast and ovarian cancers following a first breast cancer in mutation carriers were computed in an analogous manner. For these analyses, follow-up commenced at the first breast cancer or 1960 (whichever was later). Synchronous bilateral cancers and ovarian cancers occurring before a breast cancer did not, therefore, contribute to this analysis. In addition to censoring events described above for other cancers, follow-up for breast and ovarian cancers was also censored at the date of bilateral mastectomy or oophorectomy, respectively, if recorded. Estimates based on the incidence rates in all affected individuals might be biased, inasmuch as they would not account for sporadic cases. To allow for this, maximum likelihood estimates were derived by use of the program MENDEL, analogous to the procedure used for the analysis of other cancers. A separate RR was estimated for each 10-year age group 20–29 years, 30–39 years, 40–49 years, . . . 70–79 years, relative to population incidence rates. (For this purpose, we used population incidence rates averaged over all centers.) These estimates were then used to derive the cumulative risk estimates shown, with the use of the following formula:

$$F(t) = 1 - \prod_{j=0}^{t} \exp(-\mu(j)\hat{\lambda}(j)), \quad [5]$$

where $F(t)$ is the cumulative risk by age t, $\mu(j)$ is the population incidence rate of disease at age j, and $\hat{\lambda}(j)$ is the maximum likelihood estimate of RR of disease at age j.

Cumulative risks of cancers other than those of the breast and ovary were also computed by use of RRs estimated by the EM algorithm approach *(22)* described above. RRs were estimated for just two separate age groups (<65 years old and ≥65 years old) because of the smaller number of these other cancers.

Results

Risks of Cancers Other Than Cancers of the Breast and Ovary

The observed and expected numbers of cancers other than breast and ovarian cancers in the study cohort, together with the estimated RR, are shown in Table 1. Table 2 gives the corresponding RRs and 95% CIs obtained by maximum likelihood for those sites where a significant excess was observed. Significantly increased risks in carriers were observed for cancers of the stomach (RR = 2.59; 95% CI = 1.46–4.61; P = .012), pancreas (RR = 3.51; 95% CI = 1.87–6.58; P = .0012), gallbladder and bile ducts (RR = 4.97; 95% CI = 1.50–16.52; P = .03), malignant melanoma (RR = 2.58; 95% CI = 1.28–5.17; P = .01), and prostate (RR = 4.65; 95% CI = 3.48–6.22; P<.0001). There was also a statistically significant excess of cancers of other or ill-defined sites (RR = 4.13; 95% CI = 2.05–8.32; P = .01). The increased risk of cancers of the buccal cavity and pharynx did not quite reach statistical significance (RR = 2.26; 95% CI = 1.09–4.68; P = .06).

The RR of prostate cancer was significantly higher in men below the age of 65 years than in men at older ages (RR = 7.33 [95% CI = 4.66–11.52] versus RR = 3.39 [95% CI = 2.34–4.92]; P = .01). There is some suggestion that the RR was dependent on country, being lower for U.S. than for non-U.S. centers (U.S. RR = 4.28 [95% CI = 1.89–9.68] versus non-U.S. RR = 10.76 [95% CI = 6.29–18.41] below age 65; U.S. RR = 1.78 [95% CI = 0.90–3.53] versus non-U.S. RR = 5.53 [95% CI = 3.55–8.60] for age 65 years and above; P = .004).

Table 1. Observed (Obs) and expected (Exp) numbers of cancers in BRCA2 families and estimated relative risks (RRs) to BRCA2 carriers

Cancer site or type (9th ICD codes)*	Probable carriers†		Noncarriers		Unknown status		RR (95% CI) [P value]‡
	Obs	Exp	Obs	Exp	Obs	Exp	
Buccal cavity and pharynx (140–149)	4	2.26	0	1.01	8	5.74	2.26 (1.09–4.58) [.06]
Esophagus (150)	1	0.89	0	0.35	2	2.25	0.00
Stomach (151)	8	3.29	3	1.24	14	8.52	2.59 (1.46–4.61) [.012]
Colon (153)	8	6.56	6	2.85	16	14.37	1.43 (0.79–2.58)
Rectum (154)	6	3.45	4	1.47	3	8.03	1.11 (0.48–2.60)
Liver (155)	2	0.56	0	0.24	2	1.45	4.18 (1.56–11.23)
Gallbladder and bile ducts (156)	2	0.42	0	0.17	2	0.96	4.97 (1.50–16.52) [.03]
Pancreas (157)	6	2.06	0	0.87	8	4.76	3.51 (1.87–6.58) [.0012]
Larynx (161)	1	1.03	0	0.46	1	2.79	0.69 (0.11–4.37)
Lung (162)	9	11.43	4	4.79	24	27.37	1.04 (0.62–1.73)
Bone (170)	1	0.19	0	0.11	1	0.68	2.14 (0.13–36.25)
Connective tissue (171)	0	0.49	0	0.24	2	1.27	1.15 (0.07–18.56)
Malignant melanoma (172)	7	2.04	2	1.00	3	4.37	2.58 (1.28–5.17) [.01]
Cervix (180)	2	3.73	2	1.91	10	7.42	1.29 (0.48–3.43)
Other uterus (179,181,182)	5	3.35	2	1.68	2	4.99	1.25 (0.46–3.37)
Prostate (185)	29	6.06	6	2.26	40	17.09	4.65 (3.48–6.22) [<.0001]
Testis (186)	1	0.28	0	0.15	0	1.46	1.10 (0.16–7.83)
Bladder (188)	3	3.39	0	1.36	3	8.85	0.69 (0.24–1.97)
Kidney (189)	3	2.11	2	0.96	2	5.07	0.82 (0.23–2.95)
Brain (191,192)	3	1.57	1	0.79	4	4.34	1.96 (0.80–4.82)
Thyroid (193)	2	1.06	2	0.55	2	2.46	1.55 (0.43–5.53)
Hodgkin's disease (201)	2	0.82	0	0.47	1	2.62	1.48 (0.40–5.48)
Other lymphoma (200,202)	5	1.97	1	0.90	4	4.62	1.91 (0.81–4.49)
Myeloma (203)	0	0.84	0	0.35	1	1.90	0.00
Leukemia (204–208)	1	1.85	0	0.85	10	4.96	1.12 (0.30–4.25)
Other cancers§	4	1.59	0	0.74	9	3.39	4.13 (2.05–8.32) [.01]
Unknown site (199)	2	2.96	0	1.39	7	7.21	0.82 (0.22–3.15)
All cancers except breast, ovary, and non-melanoma skin	117	66.25	35	29.16	181	158.94	2.45 (2.15–2.78) [<.0001]

*Coded according to the 9th revision of the International Classification of Diseases *(14)*.

†Breast cancer case patients aged <60 years, ovarian cancer case patients and male breast cancer case patients (excluding those known to be noncarriers), and known carriers by typing and obligate carriers.

‡All P values are two-sided. CI = confidence interval.

§Three peritoneum, two other digestive, two nose, one other endocrine, two lymph node secondary, and three other/ill defined.

Table 2. Estimated relative risks (RRs) and 95% confidence intervals (CIs) for selected cancers, by age group

Site or type of cancer	0 to <65 y of age		65–85 y of age		All ages: 0–85 y	
	RR	95% CI	RR	95% CI	RR	95% CI
Buccal cavity and pharynx	1.52	0.44–5.19	3.15	1.24–7.99	2.26	1.09–4.68
Stomach	2.57	1.13–5.84	1.93	0.77–4.83	2.59	1.46–4.61
Pancreas	5.54	2.72–11.32	1.61	0.45–5.72	3.51	1.87–6.58
Gallbladder and bile ducts	*		*		4.97	1.50–16.52
Malignant melanoma	3.22	1.57–5.83	†		2.58	1.28–5.17
Prostate	7.33	4.66–11.52	3.39	2.34–4.92	4.65	3.48–6.22
All cancers except breast, ovary, prostate, and pancreas	1.48	1.15–1.91	1.30	0.96–1.76	1.47	1.21–1.79
All cancers except breast and ovary	1.89	1.52–2.33	1.72	1.36–2.17	1.90	1.63–2.23

*There were too few gallbladder cancer and bile duct cancer case patients to allow separate calculation of RRs for the two age groups unrealistic.

†Maximum likelihood procedure did not converge. There were no melanoma case patients older than 65 years among known carriers.

Among the non-U.S. centers, the RRs were higher in Iceland and Canada than in Europe (excluding Iceland), but these differences were not statistically significant.

Analyses were also conducted in which RRs for carriers and noncarriers were estimated simultaneously. There was no evidence of an overall excess of cancer in noncarriers (RR = 0.70 [95% CI = 0.50–0.99] below the age of 65 years; RR = 0.98 [95% CI = 0.75–1.27] at the age of 65 years or above). Furthermore, none of the above sites showed significantly elevated risks to noncarriers, except for prostate cancer in men below the age of 65 years (RR = 2.91; 95% CI = 1.31–6.49). This excess is largely due to three prostate cancers in close relatives in a single family in Iceland. Even in this case, allowing for an increased risk to noncarriers made little difference to the estimated risk to carriers.

Four cancers of the fallopian tube occurred in known or potential carriers; three of these cancers occurred during the follow-up period. The precise expected numbers could not be computed for this site, since cancer of the fallopian tube is grouped with ovarian cancer in the ICD. However, an analysis using rates from the East Anglian Cancer Registry suggests an approximate expected number of 0.006 (ratio of observed to expected = 500; $P<.0001$).

In addition to the cancer sites discussed above, cancers of the eye were of particular interest, since two such cancers had been previously noted in large BRCA2 kindreds *(4)*. In this dataset, three cancers of the eye were also noted, but all occurred before 1960 and hence were excluded from the cohort analysis. Of these cases, one (in an Icelandic family) occurred in a woman subsequently diagnosed with breast cancer, one (in an Irish family) occurred in an obligate carrier, and one (in a German family) occurred in a first-degree relative of a known carrier. (A further ocular cancer was reported in a Utah family, but the evidence on the site of this cancer conflicts, and this case has not been included.) Two of these cases could be included in the analysis by extending the cohort back to 1930 rather than to 1960. (The first case cannot be included in this analysis either, since it occurred before a breast cancer.) On this basis and making the assumption that incidence rates in 1960–1964 also apply to the period 1930–1959, the expected number of ocular cancers in carriers or individuals of unknown status would be 0.69 (P = .09).

Cumulative Risks

The RRs of cancer have been used to derive cumulative risks of these cancers in mutation carriers (Table 3). For pancreatic cancer, the estimated RR for males and females combined was used, since there was no evidence of any difference in RR between the sexes. For other cancers, sex-specific RRs were applied. The cumulative risks shown are derived assuming population rates for England and Wales (1988–1992) but assuming the RRs derived from the whole dataset.

If the RRs for prostate cancer derived from the whole dataset were applied to U.S. (SEER) rates, the estimated cumulative risk of prostate cancer in U.S. carriers by the age of 70 years would be 33.1% (95% CI = 26.1%–39.4%). This, however, may be a considerable overestimate, given that the RR based on U.S. families alone is somewhat lower than the overall estimate. Based on the RR obtained in U.S. families alone, the cumulative risk estimate to U.S. carriers would be 20.2% (95% CI = 11.6%–28.0%). Conversely, the cumulative prostate cancer risk to European carriers in England and Wales, based on the RR obtained in European families alone, would be 10.9% (95% CI = 4.4%–17.0%) by age 70 years.

These estimates can then be combined with previously derived breast and ovarian cancer risks to produce cumulative risks of all cancers. For ovarian and breast cancers, we used the risks derived from the BCLC families by the maximum LOD score method *(3)*. On this basis, the estimated cumulative risks for all cancers in women would be 32% by age 50 years, 56% by age 60 years, and 90% by age 70 years. Unfortunately, no precise estimate of breast cancer risk in males is currently available to our knowledge. We used the estimates derived by Easton et al. *(4)*, who estimated a cumulative risk of male breast cancer of 6% by the age of 70 years, although these estimates are based

Table 3. Estimated cumulative risks (%)* of cancers in BRCA2 mutation carriers, by sex and age

Sex	Age, y	Prostate cancer		Pancreatic cancer		Other cancer†	
		Risk	95% CI	Risk	95% CI	Risk	95% CI
Male	40	0.0	0.01–0.02	0.0	0.0–0.1	1.5	1.0–2.1
	50	0.1	0.1–0.2	0.2	0.1–0.4	3.3	2.5–4.1
	60	1.6	0.9–2.3	1.0	0.4–1.5	8.4	6.6–10.3
	70	7.5	5.7–9.3	2.1	1.2–3.0	20.2	16.9–23.4
	80	19.8	15.2–24.2	3.2	1.6–4.9	37.3	30.8–43.2
Female	40			0.0	0.0–0.1	1.9	1.2–2.6
	50			0.2	0.1–0.3	3.9	2.9–4.9
	60			0.7	0.3–1.1	8.3	6.4–10.1
	70			1.5	0.9–2.1	16.0	13.0–18.9
	80			2.3	1.1–3.5	26.0	20.2–31.3

*Cumulative risk of cancer by age t is the probability of an individual being diagnosed with cancer by their t^{th} birthday (*see* "Subjects and Methods" section). CI = confidence interval.

†"Other cancer" category consists of all cancer sites except breast, ovary, prostate, pancreas, and nonmelanoma skin cancer.

on only two large BRCA2 families. On this basis, the cumulative risk for all cancers in men would be 4% by age 50 years, 13% by age 60 years, and 32% by age 70 years.

Risk of Second Cancers

Table 4 shows the observed numbers of contralateral breast cancers and of ovarian cancers after a first breast cancer and the estimated incidence rates and cumulative risks in carriers. The estimates of the contralateral breast cancer incidence rates fall in the range of 2%–3% per year between the ages of 30 and 60 years. These risks are equivalent to a cumulative risk of breast cancer, starting at age 30 years, of 37.0% (95% CI = 25.7%–46.6%) by age 50 years, and of 52.3% (95% CI = 41.7%–61.0%) by age 70 years. The incidence rates may also be used to estimate the risk of a first breast cancer in a mutation carrier, under the assumption that the risk of cancer in the two breasts is independent, by multiplying the incidence rates by 2. The estimated cumulative risks are then 60% (95% CI = 44%–72%) by age 50 years and 77% (95% CI = 71%–88%) by age 70 years. The corresponding estimated cumulative risks of ovarian cancer were 3.3% (95% CI = 0.8%–5.7%) by age 50 years and 15.9% (95% CI = 8.8%–22.5%) by age 70 years.

Discussion

This study provides strong confirmation of an increased risk of prostate cancer and pancreatic cancer in BRCA2 mutation carriers, as well as some evidence of an excess of cancer at four other sites: buccal cavity and pharynx, stomach, melanoma of the skin, and gallbladder and bile ducts. This more general increase in cancer risk appears to contrast with the situation for BRCA1, where no excess risk was observed except for prostate cancer and colorectal cancer *(23)*. It should be emphasized, however, that the BRCA1 study was far smaller than the current study, and an RR of the order of 1.5 would not have been reliably detected. (There were only 78 cancers in carriers and first-degree relatives in that study compared with 298 in the current study.) Clearly, some of the elevated risks observed at the last four sites may have occurred by chance, given the number of cancer sites analyzed, and these associations require confirmation in other studies. There does, however, appear to be a significantly increased cancer risk in carriers, of the order of 1.5-fold, even when the sites breast, ovary, prostate, and pancreas are excluded.

An obvious concern in this study is that the observed excess cancer risk in carriers may be the result of selection of families for the occurrence of other cancers. There are several reasons for believing this to be unlikely. All centers have selection criteria for screening families based on the occurrence of breast and ovarian cancers, but not on the occurrence of other cancers. In particular, a large fraction of the data comes from large families, which would certainly have been ascertained on the basis of their breast and ovarian cancer occurrence alone. Furthermore, there is a large excess of cancer in the relatives of breast or ovarian cancer case patients who are themselves mutation carriers, but not in the relatives who are noncarriers. When RRs for carriers and noncarriers were estimated jointly, the RR for noncarriers was estimated to be slightly less than 1. The only cancer site where a statistically significant risk to noncarriers was observed was the prostate, and this excess can largely be explained by a single family in Iceland with three cases in noncarriers. Since families in Iceland are ascertained through a population-based registry, this is unlikely to be due to selection bias and is more likely to be due to coincident segregation of a prostate cancer susceptibility gene or genes in the same family.

Many of the cancers in relatives could not be typed for mutations. However, by incorporating the carrier probability of each relative into the analysis, we were able to produce unbiased RR estimates. Another potential concern is that only a proportion of cancers in relatives could be confirmed, and there is thus potential for some misclassification of cancer site. Overall, however, the excess cancer risks were similar in those centers able to confirm a high proportion of cancers (Iceland, Finland, and Sweden) than in the remainder. Misclassification of cancer site seems unlikely to have been a major problem for pancreatic cancer, prostate cancer, cancer of the buccal cavity and pharynx, or melanoma. Some of the excess of stomach cancer could be attributed to misclassification of ovarian cancer, since the observed RR was somewhat higher in female carriers than in male carriers (4.2 versus 2.1), and some of cancers of the gallbladder and bile ducts might have been misclassified pancreatic cancers.

Most of the families included in this study were selected on the basis of multiple cases of breast and/or ovarian cancer, and it is possible that the excess risks of other cancers may be different in mutation carriers with less striking family histories. At present, there are no data, to our knowledge, with which to address this issue.

The constellation of cancers associated with BRCA2 does not appear to fit any obvious pattern. Epidemiologically, breast and prostate cancers are both strongly related to endogenous sex hormones (estrogens and androgens), and both are associated with a Western-style diet. On the other hand, pancreatic cancer is not known to be associated with reproductive factors or diet, although some pancreatic tumors are estrogen receptor positive and respond to tamoxifen (as do some ovarian cancers). The strongest known risk factor for pancreatic cancer is cigarette smoking, which is also a risk factor for cancers of the buccal cavity and pharynx. However, there is no evidence of any excess risk of lung cancer in

Table 4. Observed (Obs) numbers, estimated incidence rates, and cumulative risks (95% confidence intervals [CIs]) of second (contralateral) breast and ovarian cancers, following breast cancer in BRCA2 mutation carriers

		Contralateral breast cancer			Ovarian cancer		
Age group, y	Women-years	Obs	Annual incidence rate	% cumulative risk (95% CI)*	Obs	Annual incidence rate	% cumulative risk (95% CI)
30–39	603.8	12	0.0200	17.7 (6.5–27.5)	0	0.0011	1.1 (0.0–2.2)
40–49	1127.3	25	0.0270	37.0 (25.7–46.6)	4	0.0022	3.3 (0.8–5.7)
50–59	1190.0	21	0.0200	48.4 (37.5–57.3)	8	0.0074	10.2 (4.9–15.2)
60–69	851.6	6	0.0080	52.3 (41.7–61.0)	7	0.0066	15.9 (8.8–22.5)
70–79	386.2	2	0.011	57.1 (46.4–65.6)	3	0.0063	21.0 (12.0–29.1)
Total	4158.9	66			22		

*Cumulative risks to the end of the age interval.

BRCA2 carriers. Pancreatic cancer and melanoma, but none of the other cancers, are known to occur at increased frequency in INK4A (p16) germline mutation carriers. Further detailed study of the pathology of these tumors in carriers would be worthwhile.

In terms of absolute risk, the most important effect (excluding breast and ovarian cancers) is the increased risk of prostate cancer in male carriers. This is most unlikely to be the result of increased surveillance, since most of the excess risk occurred before screening became widespread. The RR for prostate cancer was in fact higher in Europe than in the United States, where screening is more widespread; i.e., the cumulative risk of prostate cancer in U.S. carriers was lower than would be expected on the basis of the RR in Europe. This suggests that the prostate cancer risks in carriers are less affected by surveillance, which would in turn imply a different natural history, with a greater proportion of clinically detectable disease. The risk is probably not sufficiently high to cause an appreciable fraction of early-onset prostate cancer cases, except in Icelandic and Ashkenazi populations, but this needs to be studied directly. The substantially elevated risk of prostate cancer raises the issue of early detection, in that screening by prostate-specific antigen might be justified at a substantially earlier age for mutation carriers. The risk of pancreatic cancer is less important in absolute terms, although it is not insignificant in terms of mortality, since the disease is uniformly and rapidly fatal.

The analyses presented here assume a uniform risk across all mutations, and the clinical implications could be different if certain mutations were associated with higher cancer risks. There is some evidence that carriers of mutations in the central region of the BRCA2 gene, known as the OCCR (ovarian cancer cluster region), are at higher risk of ovarian cancer and, perhaps, at lower risk of breast cancer *(24)*. Analyses of genotype–phenotype associations for other cancers are in progress.

This study has also been able to provide an estimate of the risk of ovarian cancer in mutation carriers subsequent to breast cancer and of contralateral breast cancer. There is some potential for bias here, since the presence of two cancers in the same individual might have influenced the decision for a family to be referred and screened for mutations. However, none of the centers used the presence of a second cancer as part of their inclusion criteria. Moreover, the estimated risk of ovarian cancer following breast cancer is consistent with that predicted from the previous analysis of first cancers, with the current estimate being the more precise. The incidence rates for ovarian cancer are approximately fourfold lower than those for BRCA1 but, nevertheless, still more than 10-fold greater than general population rates. There is some support for the hypothesis that the ovarian cancers in BRCA2 carriers occur later than in BRCA1 carriers, although this is based on small numbers—the average incidence rates in the age group 30–49 years were sevenfold lower than those in the age group 50–69 years, whereas for BRCA1 the incidence rates were highest in the age group 40–49 years. There is even some suggestion, both from these data and from the previous estimates, that the disease occurs later than in the general population. From a practical point of view, the low rate of disease below age 50 years might indicate that prophylactic oophorectomy could be safely delayed until, say, the late thirties, and still be effective, but this needs to be tested in prospective studies. The observed risk of cancer of the fallopian tube, which is perhaps a substantial underestimate given the difficulties of determining the true primary site of these tumors, also needs to be borne in mind when considering prophylactic surgery.

The analysis of second cancers confirms, as expected, a high risk of contralateral breast cancer in affected carriers. The estimates are slightly lower than those previously derived by the BCLC for BRCA1 *(3)* (37% versus 48% by age 50 years: 52% versus 64% by age 70 years). The cumulative risk of breast cancer by age 70 years is close to what one would predict from the previously derived risks of a first cancer, by halving the incidence rates to allow for only one breast being at risk (52% observed and 60% expected), but the cumulative contralateral risk by age 50 years is significantly higher than predicted (37% observed and 15% expected). This effect (which was also seen for BRCA1) indicates either that some selection bias toward inclusion of young bilateral cases occurs or that modifying factors may be important determinants of risk at young ages.

Appendix

The following are the contributing centers and the names of the principal investigators. The number of families contributed by each center is given in **brackets:**

CRC Genetic Epidemiology Unit, Cambridge, U.K. (coordinating center): D. Easton, D. Thompson, L. McGuffog

University of Aberdeen, U.K.: N. Haites, A. Schofield [1]

Humangenetik, Kantonsspital, Basel, Switzerland: R. J. Scott [2]

Departments of Medicine and Genetics, University of Washington, Seattle: M.-C. King, E. Schubert [6]

Centre Jean Perrin, Clermont-Ferrand, France: Y. Bignon [1]

Institute of Cancer Research, London, U.K.: M. Stratton, D. Ford, J. Peto, R. Eeles [19]

CRC Human Cancer Genetics Research Group, Cambridge, U.K.: B. Ponder, S. Gayther [11]

Deutsches Krebsforschungszentrum, Heidelberg, and University of Würzburg, Germany: J. Chang-Claude, B. H. F. Weber, U. Hamann [5]

Fundación Jiménez Díaz, Madrid, Spain: J. Benitez, A. Osorio [4]

University Central Hospital, Departments of Oncology and Obstetrics and Gynaecology, Helsinki, Finland: H. Eerola, H. Nevanlinna [11]

Creighton University, Omaha, NE, and International Agency for Research on Cancer, Lyon, France: H. T. Lynch, S. Narod, D. Goldgar, G. Lenoir [8]

Institut Curie, Paris, France: D. Stoppa-Lyonnet [9]

University of Iceland, Reykjavik: A. Arason, R. Barkardottir, V. Egilsson [5]

Icelandic Cancer Society, Reykjavik, Iceland: J. Eyfjord, H. Tulinius [5]

Imperial Cancer Research Fund, Leeds, U.K.: D. T. Bishop [3]

University of Lund, Sweden: A. Borg, N. Loman, O. Johannsson, H. Olsson [20]

McGill University, Montreal, PQ, Canada: P. Tonin, W. Foulkes [11]

University of Montreal, PQ, Canada: P. Ghadirian, A. M. Mes-Masson, D. Provencher [8]

University of Pennsylvania, Philadelphia: B. Weber [11]

University of Leiden and Foundation for the Detection of Hereditary Tumours, Leiden, The Netherlands, and Erasmus Medical Center and Daniel den Hoed Cancer Center, Rotterdam, The Netherlands: P. Devilee, H. Vasen, C. J. Cornelisse, H. Meijers-Heijboer, J. G. M. Klijn [8]

University of Toronto, ON, Canada: S. Narod, J -S. Brunet, R. Moslehi [19]

University of Utah, Salt Lake City: S. Neuhausen, L. Cannon-Albright [6]

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NOTES

[1]*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

The coordination and analysis of this study has been supported by the Cancer Research Campaign and by a Concerted Action From the European Union. Data collection has been supported by the Cancer Research Campaign; by the Imperial Cancer Research Fund; by the Swedish Cancer Society; by the Foundation de France; by Biomed2 BMH4-CT96-1133; by SAF 96/0192; by the Conchita Rabago Foundation; by the Nordic Cancer Union; by the University of Iceland Hospital Research Fund; by The Cancer Society and Cancer Registry of Finland; by the Canadian Breast Cancer Research Initiative; by Public Health Service grants R01CA27632 (to M.-C. King) and CA77415 (to S. Neuhausen) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; by U.S. Army grant DAMD17-94-J-4260; and by the Utah Cancer Registry (supported by Public Health Service contract N01CN6700 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by the University of Utah).

We thank Max Parkin for assistance in obtaining cancer incidence rates for various countries. We also thank Debbie Howe for her help in preparing this manuscript.

Manuscript received November 30, 1998; revised June 2, 1999; accepted June 15, 1999.

Am. J. Hum. Genet. 64:1371–1377, 1999

Modification of *BRCA1*-Associated Breast Cancer Risk by the Polymorphic Androgen-Receptor CAG Repeat

Timothy R. Rebbeck,[1] Philip W. Kantoff,[4,5] Krishna Krithivas,[4,5] Susan Neuhausen,[6] M. Anne Blackwood,[1] Andrew K. Godwin,[3] Mary B. Daly,[3] Steven A. Narod,[7] Judy E. Garber,[4] Henry T. Lynch,[8] Barbara L. Weber,[2] and Myles Brown[4]

[1]Department of Biostatistics and Epidemiology, and [2]Medicine and Genetics, University of Pennsylvania School of Medicine, and [3]Fox Chase Cancer Center, Philadelphia; [4]Department of Adult Oncology and [5]Lank Center for Genitourinary Cancer, Dana-Farber Cancer Institute, Harvard Medical School, Boston; [6]University of Utah, Salt Lake City; [7]Women's College Hospital, Toronto; and [8]Department of Preventive Medicine, Creighton University, Omaha

Summary

Compared with the general population, women who have inherited a germline mutation in the *BRCA1* gene have a greatly increased risk of developing breast cancer. However, there is also substantial interindividual variability in the occurrence of breast cancer among *BRCA1* mutation carriers. We hypothesize that other genes, particularly those involved in endocrine signaling, may modify the *BRCA1*-associated age-specific breast cancer risk. We studied the effect of the CAG repeat-length polymorphism found in exon 1 of the androgen-receptor (AR) gene (*AR-CAG*). *AR* alleles containing longer CAG repeat lengths are associated with a decreased ability to activate androgen-responsive genes. Using a sample of women who inherited germline *BRCA1* mutations, we compared *AR-CAG* repeat length in 165 women with and 139 women without breast cancer. We found that women were at significantly increased risk of breast cancer if they carried at least one *AR* allele with ≥28 CAG repeats. Women who carried an *AR-CAG* allele of ≥28, ≥29, or ≥30 repeats were given a diagnosis 0.8, 1.8, or 6.3 years earlier than women who did not carry at least one such allele. All 11 women in our sample who carried at least one *AR-CAG* allele with ≥29 repeats had breast cancer. Our results support the hypothesis that age at breast cancer diagnosis is earlier among *BRCA1* mutation carriers who carry very long *AR-CAG* repeats. These results suggest that pathways involving androgen signaling may affect the risk of *BRCA1*-associated breast cancer.

Received October 9, 1998; accepted for publication February 18, 1999; electronically published April 5, 1999.

Address for correspondence and reprints: Dr. Timothy R. Rebbeck, Department of Biostatistics and Epidemiology, University of Pennsylvania School of Medicine, 904 Blockley Hall, 423 Guardian Drive, Philadelphia, PA 19104. E-mail: rebbeck@cceb.med.upenn.edu

0002-9297/99/6405-0016$02.00

Introduction

Inheritance of a germline mutation in the *BRCA1* gene (MIM 113705) is associated with an increased risk of developing breast cancer. However, there is also substantial variability in the ages at which breast cancers are diagnosed in *BRCA1* mutation carriers (Easton et al. 1995; Narod et al. 1995; Rebbeck 1999). These observations imply that germline mutations in *BRCA1* may be necessary to explain the Mendelian pattern of cancer in some families but may not be sufficient to completely describe the interindividual variability in the age-specific risk of cancer. The ability to effectively apply risk-prediction or cancer-prevention strategies in *BRCA1* carriers may therefore depend on knowledge of risk-modifying factors in addition to *BRCA1* mutation status.

Steroid hormone pathways regulate *BRCA1* expression (Gudas et al. 1995; Marks et al. 1997). Therefore, we hypothesize that allelic variation in genes governing hormonal signaling known to play a role in normal development and cancer risk may be involved in modification of *BRCA1*-associated cancer risk. For example, the androgen-receptor gene *AR* (MIM 313700), which functions as a ligand-dependent transcriptional activator in response to androgens, contains a highly polymorphic CAG trinucleotide repeat (*AR-CAG*) encoding glutamines in its first exon. The length of the *AR-CAG* polymorphism is inversely associated with the degree of transcriptional activation by the AR (Chamberlain et al. 1994; Kazemi-Esfarjani et al. 1995). Individuals with X-linked spinal and bulbar muscular atrophy (SBMA, Kennedy disease) have 40 or more *AR-CAG* repeats and manifest clinical androgen insensitivity (LaSpada et al. 1991). *AR* mediates breast tumor growth and progression (Zhu et al. 1997; Birrell et al. 1998). Increased *AR-CAG* repeat length has also been associated with decreased prostate cancer risk, presumably because of a decreased ability of androgens to stimulate transcription of genes involved in prostate growth (Hardy et al. 1996; Giovannucci et al. 1997; Ingles et al. 1997). However,

this result has not been replicated in all populations (Eeles et al. 1998). These findings suggest that *AR-CAG* repeat-length polymorphism may be involved in modifying the development of diseases caused by alterations in endocrine signaling.

Subjects and Methods

Subjects

A sample of 304 women who carry disease-associated germline *BRCA1* mutations was ascertained through families with a history of breast and/or ovarian cancer at Creighton University, the Dana Farber Cancer Institute, The University of Michigan, Fox Chase Cancer Center, The University of Pennsylvania, The University of Utah, and Women's College Hospital (Toronto) between 1978 and 1997. Women were self- or physician-referred because of a strong family history of breast and/or ovarian cancer. These women provided written informed consent for research under protocols approved by the institutional review boards at each institution. Of these 304 women, 165 (54%) were affected (mean age 40.5 years, range 21–73 years) and 139 (46%) were unaffected by breast cancer (mean age 44.5 years, range 19–89 years).

Genotype Analysis

All study participants provided peripheral blood samples from which genomic DNA was extracted according to standard protocols. We used PCR to amplify the *AR-CAG* trinucleotide repeat found in exon 1 of *AR*, as described elsewhere (Giovannucci et al. 1997). The *AR-CAG* repeat-length polymorphism was modeled in three ways to reflect two alternative hypotheses about the activity of *AR* in breast carcinogenesis. The first two models reflect the activity of specific alleles acting at the level of the breast epithelial cell to modulate androgen signaling. First, the shorter of the two repeat-length alleles for each subject was considered in a survival analysis model. For example, if an individual inherited alleles with 22 and 25 *AR-CAG* repeats, we considered the effect of the 22-repeat allele on breast cancer penetrance. This allowed us to evaluate whether having at least one short-repeat allele affected breast cancer penetrance. Second, the longer of the two repeat-length alleles for each subject was considered in the survival model. As before, this allowed us to evaluate whether having at least one very long allele affected breast cancer penetrance. The third model reflects the effect of the combined *AR* genotype as having endocrine or paracrine activity on levels of steroid hormones in breast carcinogenesis. This hypothesis was modeled by consideration of the mean allele repeat length as a measure of jointly considering both alleles in each individual.

Statistical Methods

Cox proportional hazards models were used to evaluate the difference in breast cancer penetrance across *AR-CAG* repeat lengths. To correct for nonindependence of observations among participants drawn from the same families, the robust variance-covariance estimation approach of Lin and Wei (1989) was used, as implemented in STATA (StataCorp., release 5). Participants were followed up (retrospectively) from birth until one of several events occurred. The primary event of interest was the first diagnosis of a primary invasive breast cancer (n = 165 54%). Participants with no prior breast cancer diagnosis were censored when they developed ovarian cancer (n = 40; 13%), had a prophylactic mastectomy or oophorectomy (n = 46; 15%) or died (n = 15; 5%)—or when none of these events had occurred by the end of the observation period (n = 38; 13%). All Cox proportional hazards analyses were undertaken with and without adjustment for three hormone-related risk factors: age at menarche, age at first live birth, and total number of full-term pregnancies (parity). Parity and age at menarche are the only factors on this list that have been previously suggested as modifiers of breast cancer risk in *BRCA1* carriers (Narod et al. 1995).

AR-CAG repeat length is known to be correlated with endocrine signaling. However, the *AR-CAG* repeat is continuously distributed, and there is no a priori point at which cutoffs may be applied to identify allele-specific risk groups. Therefore, sensitivity analyses were undertaken to compare *AR-CAG* genotype classes by use of the log rank statistic estimated from Kaplan-Meier models. Risk (hazard) ratios were estimated by using Cox proportional hazards models. These analyses involved dichotomizing the total sample by using cutpoints along the *AR-CAG* repeat-length distribution to compare women whose *AR-CAG* repeat allele was less than, greater than, or equal to the specified number of repeats. Cutpoints were made within the range of observed *AR-CAG* repeat lengths. First, the effect of having at least one very short allele was evaluated by comparing groups divided at repeat lengths $<15/\geq 15$ through $<25/\geq 25$, where the repeat-length cutpoint was determined by the shorter of a woman's two *AR* alleles. Analyses were undertaken in this range because few alleles with <14 *AR-CAG* repeats were observed. The 25-repeat allele was used as the upper cutpoint bound, because few shorter alleles with >25 repeats were observed. Second, the effect of having at least one very long allele was evaluated by comparison of groups divided at allele lengths $<20/\geq 20$ through $<30/\geq 30$, where the repeat-length cutpoint was determined by the longer of a woman's two *AR* alleles. The 30-repeat *AR-CAG* allele was used as the upper bound, because few 31- or 32-repeat alleles were observed in this sample (fig. 1). This

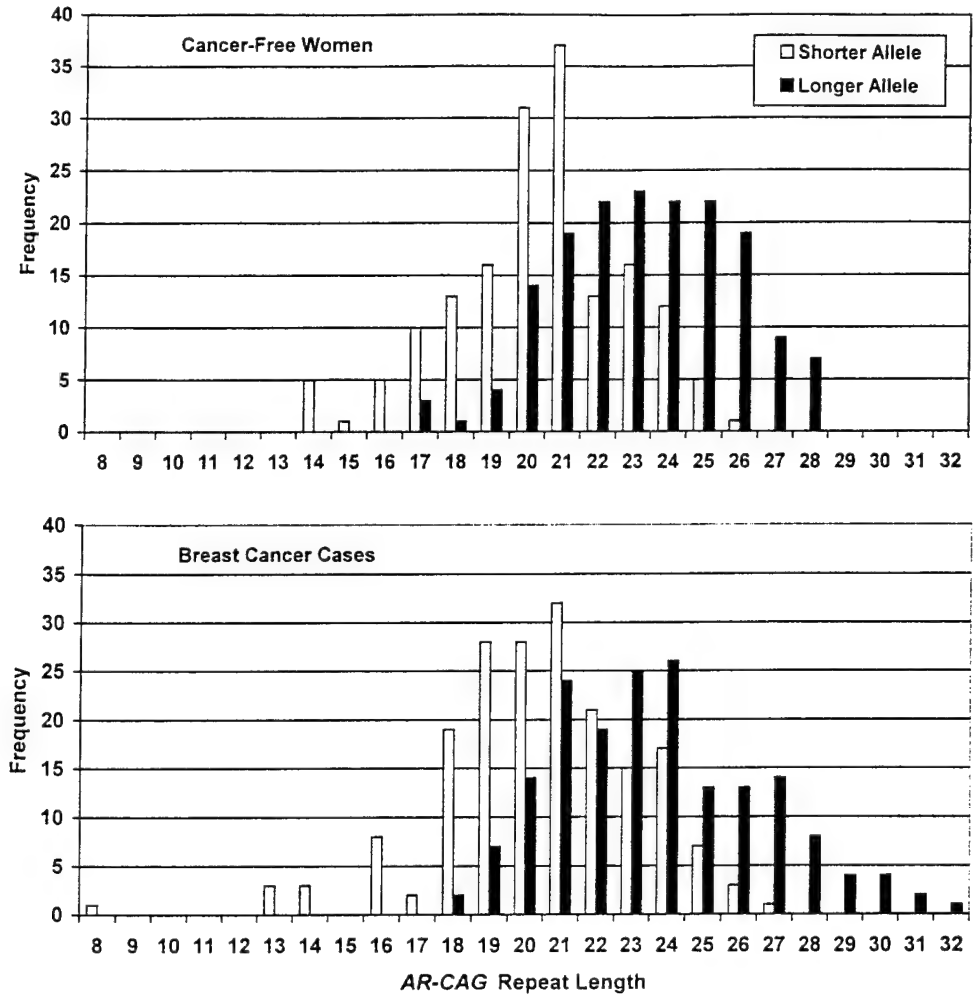

Figure 1 *AR-CAG* repeat-length distribution by breast cancer status

analysis allowed us to identify critical cutpoints along the continuous allele distribution for which breast cancer penetrance may be modified.

Results

We evaluated the effect of *AR-CAG* repeat length on breast-cancer penetrance in 304 women who carry germline mutations in the *BRCA1* gene. Mutations in *BRCA1* spanned the majority of the gene's coding region (ranging from Met1Ile to 5439delA). Mutations included 198 (65%) deletions (including large genomic deletions), 55 (18%) nonsense mutations, 31 (10%) insertions, and 20 (7%) disease-associated missense mutations. The three most commonly identified mutations were 185delAG (n = 49), Q1313ter (n = 33), and 5382insC (n = 26).

A frequency histogram depicting the observed distribution of *AR-CAG* repeat lengths is presented in figure 1. Although the distributions among cancer patients and cancer-free women are generally similar, we observed a skew in the distribution of breast cancer cases toward inheritance of at least one longer-repeat allele compared with cancer-free women. The median repeat length was 22 (range 8–32). The overall allele repeat distribution in our sample (including mean, median, and range) is similar to that of control populations reported elsewhere (Giovannucci et al. 1997; Hakimi et al. 1997). The median repeat length of the shorter allele carried by each individual was 21 (range 8–30). The median repeat length of the longer allele carried by each individual was 24 (range 17–32).

We found no simple association between breast cancer penetrance and continuous CAG repeat variables coded as mean repeat length (RR = 1.03, 95% CI 0.96–1.10), shorter repeat length (RR = 1.01, 95% CI 0.95–1.07), or longer repeat length (RR = 1.04, 95% CI 0.98–1.10). To identify points in the continuous *AR-CAG* repeat-length distribution associated with modified breast cancer penetrance, we performed sensitivity analyses using the shorter or longer of a woman's two alleles. No effect

of a woman's shorter *AR* allele was observed (fig. 2). In contrast, women who carried at least one long *AR-CAG* repeat allele (≥28 repeats) had a significantly earlier age at breast cancer diagnosis than women without one long-repeat allele (fig. 3). In the group of women who carried at least one allele with ≥28 repeats, we observed eight breast cancers (mean age at diagnosis 41.0 years) and four unaffected women (mean age at diagnosis 37.5 years) with 28-repeat alleles, four breast cancers (mean age at diagnosis 40.8 years) in women with 29-repeat alleles, and seven breast cancers (mean age at diagnosis 34.0 years) in women with ≥30-repeat alleles. There were no women in this study who carried two alleles with >28 repeats.

Estimates of hazard ratios (HRs) from Cox proportional hazards models indicated an increasing breast cancer penetrance as the repeat-length cutpoint increased (fig. 3). Women with ≥28 *AR-CAG* repeats (n = 19) developed breast cancer 0.8 years earlier than women who had only shorter alleles (n = 146; HR = 1.81, 95% CI 1.06–3.08). Similarly, women with ≥29 *AR-CAG* repeats (n = 11) developed breast cancer 1.8 years earlier than women who had only shorter alleles (n = 154; HR = 2.66, 95% CI 1.51–4.69), and women with ≥30 *AR-CAG* repeats (n = 7) developed breast cancer 6.3 years earlier than women who had only shorter alleles (n = 158; HR = 4.45, 95% CI 1.31–15.16). As indicated in figure 1, all women in our sample who carried at least one allele with ≥29 repeats were affected. All estimates presented here were made without adjustment for other potential confounding variables. However, inferences from survival analyses comparing *AR-CAG* repeats on breast cancer penetrance were identical in analyses that were unadjusted and adjusted for parity, age at menarche, age at first live birth, or ascertainment site. Note that the 24 individuals who carried at least one *AR-CAG* allele of ≥28 repeats included 20 unrelated women and two pairs of relatives (i.e., two relatives in each of two families, each pair carrying the same mutation). Five of the unrelated women in this group carried the same *BRCA1* mutation (185delAG).

It was not possible to conduct a complete evaluation of the effect of *BRCA1* mutation type given the extreme heterogeneity in the type and location of *BRCA1* mutations. However, we repeated our analyses including only women with at least one *AR-CAG* allele of ≥28 repeats by using a sample of women with unique *BRCA1* mutations. Our inferences were similar to those presented in figure 3: having at least one allele with 28 or more *AR-CAG* repeats (HR = 3.7, 95% CI: 1.6–8.4), 29 or more *AR-CAG* repeats (HR = 3.6, 95% CI: 1.3–9.8), or 30 or more *AR-CAG* repeats (HR = 4.5, 95% CI 1.1–18.1) remained significantly associated with breast cancer risk.

Discussion

We report that *AR-CAG* allele size is associated with breast cancer penetrance in *BRCA1* mutation carriers. Although the present association study is primarily hy-

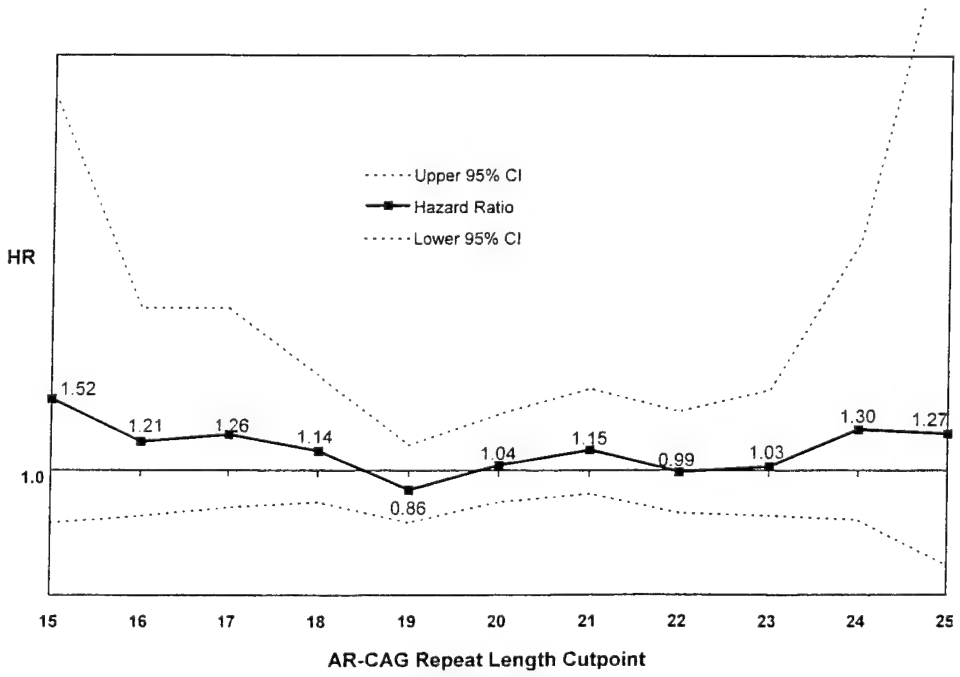

Figure 2 HRs associated with shorter *AR-CAG* repeat length among 304 *BRCA1* mutation carriers

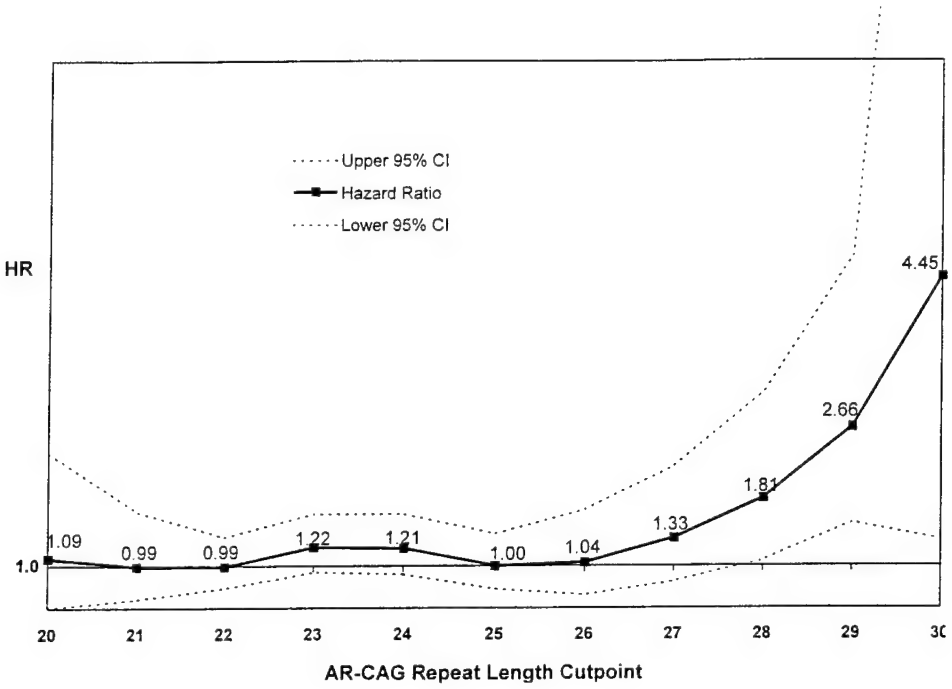

Figure 3 HRs associated with longer *AR-CAG* repeat length among 304 *BRCA1* mutation carriers

pothesis generating, there is a strong biological rationale for our findings. It is well known that *AR-CAG* repeat length modulates the transactivational activity of *AR* in vitro and is inversely associated with androgen sensitivity (LaSpada et al. 1991; Chamberlain et al. 1994; Kazemi-Esfarjani et al. 1995; Tut et al. 1997). The *AR* is expressed in normal breast epithelial cells and in some breast tumors, and it may be coexpressed with the estrogen and progesterone receptors in breast tumors (Hackenberg and Schulz 1996). Androgens are known to inhibit the growth of some breast cancer cell lines (Birrell et al. 1995). Ectopic *AR* expression leads to the inhibition of breast tumor–cell proliferation in response to androgens (Szelei et al. 1997). Although side effects have limited their use, androgens are effective in the treatment of women with metastatic breast cancer (Goldenberg et al. 1973). This information supports the hypothesis that decreased androgen activity associated with an increased number of *AR-CAG* repeats may result in increased breast cancer risk.

One means by which *AR* may act in breast tumorigenesis is by androgen signaling acting on the level of the mammary epithelial cell. Since *AR* maps to the X chromosome, breast epithelial cells in women express only one of the two *AR* alleles a woman has inherited. Thus, each cell is under the influence of only a single *AR* allele. Acting on knowledge of *AR* activity in breast cell proliferation, we hypothesize that decreased androgenic activity in breast cells expressing a very long *AR-CAG* repeat allele may result in increased breast epithelial cell proliferation. This increased proliferation may in turn affect the penetrance of breast cancer in *BRCA1* mutation carriers. In support of this hypothesis, Elhaji et al. (1997) have reported somatic mutations leading to a significant lengthening of the *AR-CAG* repeats in breast tumors from postmenopausal women. Thus, our finding of a relationship between the longer *AR-CAG* allele and breast cancer risk in *BRCA1* mutation carriers supports a model in which the effect is mediated at the level of the mammary epithelium in a cell-autonomous fashion.

An alternative to this hypothesis is an endocrine or paracrine mechanism for the action of *AR* in breast tumorigenesis. Androgens may modulate *BRCA1* risk via an endocrine mechanism by altering the levels of circulating hormones, such as estradiol, or via a paracrine mechanism involving effects mediated by the mammary stroma. For example, a recent epidemiologic study of the relationship between testosterone and breast cancer risk reported that decreased serum testosterone levels may have an indirect effect by influencing the bioavailability of estrogen (Zeleniuch-Jaquotte et al. 1997). Alternatively, androgens might act on the mammary stroma to indirectly affect the growth of the mammary epithelium. If either of these indirect mechanisms is mediating the effect of the *AR* polymorphism on *BRCA1* risk, the effect would be expected to be the result of the action of the *AR* in a number of cells, rather than in a cell-autonomous fashion, and thus would reflect the activity of both *AR* alleles. We modeled this effect of both

AR alleles as the average of the signal from the two alleles, and we found no support for this hypothesis. However, our ability to reject these hypotheses was limited, and we cannot rule out the possibility that endocrine or paracrine effects of androgens may affect breast cancer risk in *BRCA1* mutation carriers.

One limitation of the present study is that the participants carried a variety of *BRCA1* mutations, and we could not evaluate the effect of *BRCA1* mutation type or location on the present results. However, it is unlikely that the heterogeneity of *BRCA1* mutations affected the inferences of this study. Previous reports suggest that the location of the *BRCA1* mutation may, in part, determine breast versus ovarian cancer risk (Gayther et al. 1995). The data available from large consortia indicate that no differences exist in breast cancer risk by mutation location or type (D. Easton, personal communication). The common Ashkenazi Jewish mutations in *BRCA1* (i.e., 185delAG and 5382insC) confer approximately the same lifetime breast cancer risk (Struewing et al. 1997). In addition, the effect of *AR-CAG* repeat length among women who carried at least one longer allele of ≥28 repeats remained, even after limiting the sample to women who had unique *BRCA1* mutations. This suggests that *BRCA1* mutation type did not artificially induce the association of *AR-CAG* alleles and breast cancer risk. Given the extreme heterogeneity of mutations in *BRCA1*, it is unlikely that a comprehensive analysis of the effect of mutation location or type could be done. Furthermore, because the analyses were not limited to a particular class of mutations, the present results may be applicable to the general population of *BRCA1* mutation carriers from high-risk families. An additional limitation is that some individuals in the families studied here may have been excluded because they had died or were otherwise unable to participate in this research. As a result, the present results do not allow us to determine whether this effect implicates *AR* as an independent breast cancer risk factor or as a modifier of *BRCA1*-associated breast carcinogenesis.

Our results suggest that female *BRCA1* mutation carriers who have inherited at least one very long *AR-CAG* repeat may be diagnosed with breast cancer at a significantly earlier age than women who do not carry a very long *AR-CAG* repeat. Because the frequency of long *AR-CAG* repeats is rare, the *AR-CAG* repeat polymorphism may be relevant only to some *BRCA1* mutation carriers. However, the large magnitude of effect suggests that the personal impact on breast cancer risk to women who carry the very long repeats could be substantial. We conclude that the length of the *AR-CAG* repeat may affect the timing of breast cancer diagnosis in *BRCA1* mutation carriers, possibly through modulation of hormonal responses of individual mammary epithelial cells. However, these results are preliminary, and it is premature to consider knowledge about *AR-CAG* genotype in making clinical decisions about breast cancer risk, surveillance, or prevention among *BRCA1* mutation carriers.

Acknowledgments

The authors wish to thank Andre Rogatko for his helpful discussions of the statistical analyses. This study was supported by grants from the Public Health Service (ES08030 and CA737370 [to T.R.R.], CA57601 [to B.L.W.], CA70328 [to A.K.G.], and CA55914 and CA74415 [to S.L.N.]), the University of Pennsylvania Cancer Center (to T.R.R. and B.L.W.), The Breast Cancer Research Foundation (to B.L.W.), the Dana-Farber Women's Cancers Program (to J.E.G. and M.B.), the Dana-Farber Prostate Cancer Research Fund (to P.W.K. and K.K.), the Department of Defense (DAMD-17-96-I-6088 [to A.K.G.], DAMD-17-94-J-4425 [to M.D.B.], DAMD17-94-J-4260 [to S.L.N.], and DAMD-17-94-J-4340 and DAMD-17-97-I-7112 [to H.T.L.]), The Utah Cancer Registry (funded by Public Health Service Grant NO1-CN-6700) and the Utah State Department of Health, and the Nebraska State Cancer and Smoking-Related Diseases Research Program (LB595 [to H.T.L.]).

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/index.html (for *BRCA1*)

Genome Database, http://www.gdb.org/ (for *BRCA1* and *AR*)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *BRCA1* [MIM 113705] and *AR* [MIM 313700])

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American Cancer Society Second National Conference on Cancer Genetics

Supplement to Cancer

Ethnic Differences in Cancer Risk Resulting From Genetic Variation

Susan L. Neuhausen, Ph.D.

Division of Genetic Epidemiology, Department of Medical Informatics, University of Utah School of Medicine, Salt Lake City, Utah.

Ethnic differences in cancer incidence and mortality exist and are probably the result of genetic and epidemiological risk factors. Genetic differences caused by founder mutations are reviewed, with special emphasis on mutations in *BRCA1* and *BRCA2*. Germline mutations in cancer susceptibility genes have been identified in individuals of all races and ethnic groups. Differences among ethnic groups for cancer risks have been recognized, and a proportion of the differences may be the result of founder mutations within these genes. The *BRCA2* 999del5 mutation in Iceland and the three *BRCA1* and *BRCA2* mutations in Ashkenazic Jews have been well characterized and were easy to study because the patient population and anonymous samples were readily available and ethnicity was known. Mutations in *BRCA1* and *BRCA2* probably account for approximately 3 to 10% of breast cancer in the general population and a much higher proportion in those with a strong family history of breast and ovarian cancers and in those of Ashkenazic Jewish descent. However, no overall increased risk of breast or ovarian cancers exists among Ashkenazic Jewish women compared with non-Jewish Caucasians. Some ethnic variation in cancer risk may be explained by founder mutations identified in cancer-predisposing genes. Knowledge acquired by studying the effect of a single mutation in a well defined population may be applied to larger, more heterogeneous populations. Individuals from all racial and ethnic groups carry deleterious mutations. Mutations are simply easier to find and characterize when identified in a specific ethnic group. *Cancer* 1999;86:000–000.

KEYWORDS: genetic variation, germline mutations, *BRCA1*, *BRCA2*, cancer risk estimates

Cancer is caused by both exogenous and endogenous factors. The published probabilities of developing cancer are averages across the population. They do not factor in individual behavior and risk factors. Demographic factors include age, sex, race, socioeconomic status, and geographic location.[1] With increasing age, there is an increased risk for many cancers, including breast and prostate cancers. Sex is a risk factor for some cancers because some are sex-limited (e.g., ovarian and prostate cancers), and others are more common in one sex; e.g., breast cancer is 100 times more common in women than in men.

Other risk factors include exposure to physical and biologic agents (chemical exposures, drugs, infectious agents,and so forth), which may increase risks of certain cancers such as lung and gastric cancers.[1] Lifestyle factors, including alcohol use, smoking, diet, and exercise, may also affect cancer risk.[1] For breast cancer, known reproductive factors such as age at menarche and menopause, age at

Presented at the American Cancer Society Second National Conference on Cancer Genetics, San Francisco, CA, June 26–28, 1998.

Supported by grants from the National Cancer Institute (R01 CA-74415) and the Department of Defense (DAMD17-94-J-4260 and DAMD17-96-1-6266).

Address for reprints: Susan L. Neuhausen, Ph.D., Genetic Epidemiology, 391 Chipeta Way, Suite D 2, Salt Lake City, UT 84108.

Received May 25, 1999; accepted June 3, 1999.

TABLE 1
Examples of Founder Mutations Identified In *BRCA1* and *BRCA2*

Population	Mutations	References
Ashkenazic Jews	*BRCA1*-185delAG, 5382insC; *BRCA2*-6174delT	Struewing et al.,[36] Tonin et al.,[37] Neuhausen et al.[38]
Icelanders	*BRCA2*-999del5	Thorlacius et al.[28]
Dutch	*BRCA1*-2804delAA, del510, del3835; *BRCA2*-5573insA	Peelen et al.,[31] Petrij-Bosch et al.[32]
Norwegians	*BRCA1*-1136insA	Andersen et al.[29]
Swedes	*BRCA1*-Q563X, 3166ins5, 1201del1, 2594delC; *BRCA2*-4486delG	Johannsson et al.,[27] Hakansson et al.[39]
African Americans	*BRCA1*-M1775R, 1832del5, 5296del4	Gao et al.[35]

first pregnancy, number of full-term pregnancies, and oral contraceptive use are important.[2,3] One of the largest risk factors is a family history of cancer. Relative risks range from 2 to 9 depending on the type of cancer, age, and number of first-degree relatives affected by the disease.[1] Segregation analyses of pedigrees often suggest a genetic basis for the family history.

Ethnic differences in cancer incidence and mortality are well documented.[4] For example, African-American men have the highest incidence of prostate cancer and Japanese men living in Japan have the lowest incidence.[5-7] With migration to the United States, the rate of prostate cancer increases in Asians,[8] suggesting that diet or lifestyle factors contribute to development of the disease.[9] It also has been hypothesized that a portion of the observed ethnic differences in cancer susceptibility may be explained by genetic factors from mutations in rare genes that confer high risk[10,11] and/or from alleles of specific genes that confer modestly increased risk, such as androgen metabolism genes.[12,13] Clear ethnic differences have also been observed in breast cancer populations. Hispanic and Native American women have the lowest incidence of breast cancer compared with non-Hispanic Caucasians and African Americans.[4] Hypotheses regarding lifestyle, reproductive, and screening factors explain some of the differences in breast cancer incidence.[14] However, the ways that different risk factors specifically act and interact to promote cancer are largely unknown.

An endogenous factor that must be considered is the role of inherited (germline) mutations in ethnic differences in cancer risk. A genetic predisposition probably accounts for approximately 5 to 10% of cancer. Genes for more than 20 cancer syndromes have been identified. Differences among ethnic groups for cancer risks in some of these genes have been recognized and are caused by a common germline mutation within an ethnic group.

Ethnic differences may arise from founder effects, which occur when a population is established by a small number of people. Once the population expands, the mutation in one of the founders then becomes prevalent in a larger proportion of the population. The evolutionary significance of founder effects can be studied by following pedigrees for many generations and examining genetic relationships. Examples of populations in which founder effects are well documented include Afrikaners of South Africa,[15] Finns,[16] Ashkenazic Jews,[17] and French Canadians.[18] Examples specific to cancer genes are a founder mutation in *APC* found in Ashkenazic Jews,[19] one in *hMLH1* found in Finns,[20] one in *VHL* found in Germans,[21] one in *CDKN2* found in Dutch,[22] and mutations found in *BRCA1* and *BRCA2* in many different groups.[23] This review focuses on founder mutations identified in *BRCA1* and *BRCA2*, two genes that predispose individuals primarily to breast and ovarian cancers.

For *BRCA1* and *BRCA2*, more than 300 mutations have been identified in individuals of all racial and ethnic groups.[24,25] As DNA from individuals is evaluated, recurring mutations are identified. These are then further examined to determine if they are founder mutations (e.g., a shared haplotype) or ones that arose two or more times by chance. Founder mutations for *BRCA1* and *BRCA2* have been described in French Canadians,[26] Swedes,[27] Icelanders,[28] Norwegians,[29] Finns,[30] Dutch,[31,32] Russians,[33] Japanese,[34] African Americans,[35] and Ashkenazic Jews.[36-38] A partial list of mutations is presented in Table 1.

Complex and controversial issues that arise from genetic research pertain to who should be offered predictive testing and when it should be done. An important consideration for testing is the probability that an individual with breast or ovarian cancer (or both) will have a mutation in *BRCA1* or *BRCA2*. Estimates are that the gene frequency of a major gene(s) for breast cancer is 0.0033[40] and of *BRCA1* is 0.0006,[41] so that the likelihood of an individual carrying a mutation is low. Many studies have been performed to identify mutation prevalence and to develop probability models to predict a mutation carrier before testing.

TABLE 2
Prevalence of the Icelandic *BRCA2* Founder Mutation 999del5

Group	No.	No. of occurrences	Comments	References
Breast cancer population-based	520	3 (0.6%)		Thorlacius et al.[42]
Families	21	16 (76.0%)	9/16 had male breast cancer	Thorlacius et al.[28]
Male breast cancer cases	30	12 (40.0%)	In the 9 families	Thorlacius et al.[28]
Female breast cancer cases	632	49 (7.7%)		Thorlacius et al.[42]
Population-based prostate cancer	65	2 (3.1%)	Significantly worse survival	Sigurdsson et al.[43]

TABLE 3
Frequency of *BRCA1* and *BRCA2* Mutations in Ashkenazic Jews

Source	No.	185delAG	5382insC	6174delT	References
Population-based	varied	0.8–1.1%	0.13–0.3%	0.9–1.5%	Struewing et al.,[36] Roa et al.,[45] Oddux et al.[46]
BC < age 42	80	20.0%	4.0%	8.0%	Neuhausen et al.,[38] Offit et al.[47]
BC 42–50 yrs	27	30.0%	4.0%	7.0%	Neuhausen et al.,[38] Offit et al.[47]
BC only families	138	20.0%	5.0%	4.0%	Tonin et al.[48]
B/O families	82	52.0%	16.0%	5.0%	Tonin et al.[48]

BC: breast cancer; B/O: breast and ovarian cancers.
Reprinted from *Genetic Testing* 1997; 1:75–83.

Much more information is available for the *BRCA2* 999del5 mutation in Icelanders and the three founder Ashkenazic Jewish mutations because a large number of samples are available. In addition, mutation detection is rapid and inexpensive compared with screening entire genes.

The population prevalence and proportion of individuals with breast, ovarian, and prostate cancer with the *BRCA2* 999del5 mutation in the Icelandic population are shown in Table 2. This mutation in Iceland is approximately 20 times more prevalent (0.6%)[42] than the estimated allele frequency in the general population.[40] In Icelandic breast cancer cases unselected for a family history, it accounts for 7.7% of female breast cancer diagnosed at any age and for 24% of those diagnosed in women younger than 40 years.[42] It also was the cause of disease in the majority (76%) of high-risk breast cancer families studied.[28] For males, it accounts for 40% of male breast cancer and 3.1% of prostate cancer.[42, 43] The risk ratio of prostate cancer in first-degree relatives of mutation carriers is 4.6.[43] This mutation with the same haplotype has also been seen in Finland.[30,44]

Table 3 is a similar table for the three common mutations identified in Ashkenazi Jewish breast and ovarian cancer patients. The population prevalence for these three mutations combined is 2 to 2.5%,[36,45,46] which is approximately 10 to 50 times higher than the allele frequency in the general population. Based on a number of studies, approximately 30% of breast cancer diagnosed in those younger than 40 years and 39% of ovarian cancer diagnosed in those younger than 50 years in this population are caused by one of the three founder mutations.[49–51] Therefore, even in the absence of a strong family history, Ashkenazic Jewish women with breast or ovarian cancers have a much higher probability than do non-Jewish women of being *BRCA1* or *BRCA2* mutation carriers. However, even though mutations in these genes are more common in Ashkenazic Jewish women, there is little to no overall increased risk of breast or ovarian cancers among these women compared with non-Jewish Caucasians.[52] Egan et al.[52] reported a suggestion of an increased risk of breast cancer in Jewish women with a family history, which could reflect the frequency of the founder *BRCA1* and *BRCA2* mutations.

In general, mutations in both *BRCA1* and *BRCA2* in one individual are rare, given the frequency of mutations. In the Ashkenazic Jewish population, the *BRCA1* 185delAG and *BRCA2* 6174delT both occur with frequencies of 1%, so it is not surprising that several reports have been made of Jewish women with both *BRCA1* and *BRCA2* mutations[53,54] (Neuhausen, unpublished data). Although these women are carrying two deleterious mutations, age of onset of cancer and prognosis do not appear to be different than in those with only one mutation.

The focus on the "Jewish" mutations has caused

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concern in the Jewish community that *BRCA1* and *BRCA2* are peculiar to the Jewish people.[55,56] But that is not the case. *BRCA1* and *BRCA2* mutations have been identified in individuals of all racial and ethnic groups. As referenced above, relatively homogenous populations have founder mutations in which small genetic alterations that cause disease are easy to find. These groups (e.g., Ashkenazic Jews, French Canadians, Finns, Afrikaners) are then the first to be studied because information obtained from studying the effect of a mutation in a well defined population may be beneficial for determining effects in larger, more heterogeneous populations. The *BRCA1* and *BRCA2* mutations in Ashkenazic Jewish populations were easy to study because the patient population and anonymous samples from prenatal testing were readily available and identified as being of Jewish ancestry. Ashkenazic Jews do not have more defective DNA than any other ethnic group does, and they do not have higher rates of hereditary diseases than others. The same is true for other ethnic groups in which founder mutations have been identified.

It is estimated that in the general population, approximately 6 to 7% of breast cancer cases and 10% of ovarian cancer cases averaged across all ages of onset result from mutations in breast cancer susceptibility genes.[57] The frequency of *BRCA1* and *BRCA2* mutation carriers in women with breast or ovarian cancer (or both) depends on the study population.

In a large clinic-based study, the minimum criterion for entry was breast cancer at younger than 50 years or ovarian cancer at any age and a minimum of one affected first-degree or second-degree relative with breast cancer younger than 50 years or ovarian cancer at any age.[58] Mutations in *BRCA1* or *BRCA2* were detected in 45% (50 of 101) of women with at least two affected relatives and in 22% (20 of 89) of women with only one affected relative.[58] In non-Jewish women with breast or ovarian cancer (or both)and a family history of breast and/or ovarian cancer, the risk of carrying a mutation in *BRCA1* and *BRCA2* was approximately the same as in Jewish women (38.7% and 42.6%, respectively).[58] The presence of a strong family history of disease was a significant predictor of the likelihood of carrying a *BRCA1* or *BRCA2* mutation.

The results from this cohort of breast and/or ovarian cancer cases with a strong family history can be compared with those of other clinic-based studies and those of population-based studies. In two clinic-based studies that selected women based exclusively on age of onset as a predictor of *BRCA1* status, 8% and 10% of women younger than 35 and 30 years, respectively, were found to carry germline mutations in the *BRCA1* gene.[59,60] In a population-based study, Malone et al.[61] reported that of 208 Caucasian women diagnosed with breast cancer before their 45th birthdays who had a family history of breast cancer in first-degree relatives, 15 (7.2%) had germline mutations in *BRCA1*. In this study, the younger the age at diagnosis of cancer and the stronger the family history, the higher the percentage of mutations found. In another population-based study, Newman et al.[62] reported that *BRCA1* mutations were found in only 3.3% (4 of 120) of Caucasian women with breast cancer diagnosed between ages 20 and 74 years. Family history was the greatest predictor of *BRCA1* mutation status, based on both number of affected relatives and presence of ovarian cancer in a relative.[62] The conclusion from these studies is that the stronger the family history of breast and/or ovarian cancer and to a lesser extent, the younger the age at diagnosis, the more likely a breast or ovarian cancer case is to carry a mutation in *BRCA1* or *BRCA2*.

Most breast cancer studies have examined women of Northern European ancestry. African American women, who have a higher incidence of early onset breast cancer,[4] have yet to be studied extensively. One can infer from the available data for *BRCA1* that mutations in African American differ from those in Caucasians and that there also may be founder effects in this population. Three novel *BRCA1* mutations were identified in five of nine (56%) African-American families screened for mutations.[35] In the population-based study of Newman et al.,[62] no mutations were identified in 99 African-American women with breast cancer. This suggests that, as in Caucasians, the incidence of *BRCA1* mutations in African Americans is most likely to occur in patients with a strong family history of breast cancer and a young age at diagnosis.

Models have been developed to predict the likelihood that a woman has a germline *BRCA1* or *BRCA2* mutation.[58,63-66] In two separate studies, researchers at the University of Pennsylvania (Philadelphia, PA)[63] and at Myriad Genetic Laboratories (Salt Lake City, UT)[64] screened for mutations in *BRCA1* then used logistic regression analysis to develop models to evaluate the probability of a woman carrying a deleterious mutation. For the model developed by Couch et al.,[63] the predicted probability is the same for a woman with breast or ovarian cancer and for her family. Regression variables included age at diagnosis, family history of breast and ovarian cancer, both breast and ovarian cancer in a single family member, and Ashkenazic Jewish descent. The model developed by Shattuck-Eidens et al.[64] included the above variables as well as the type of cancer and number of affected relatives. In a recent analysis, Frank et al.[58] calculated the probability that a woman with breast and/or ovarian cancer who has a strong family history of breast and ovarian

TABLE 4
Estimated Cumulative Risks of Developing Breast and Ovarian Cancers

Breast cancer by age	BCLC-*BRCA1*[68]	BCLC-*BRCA2*[69]	Ashkenazi-*BRCA1/BRCA2*[70]	Population w/BRCA#[40]	General population[1]
30	0.036 (0–0.14)	0.006 (0–0.019)	0	0.017	0.0002
40	0.18 (0–0.35)	0.12 (0–0.24)	0.15 (0.07–0.23)	0.144	0.005
50	0.49 (0.28–0.64)	0.28 (0.09–0.44)	0.33 (0.23–0.44)	0.376	0.01
60	0.64 (0.43–0.77)	0.48 (0.22–0.65)	0.54 (0.38–0.68)	0.548	0.02
70	0.71 (0.53–0.82)	0.84 (0.43–0.95)	0.56 (0.40–0.73)	0.647	0.04
Ovarian	0.42	0.27 (0–0.47)	0.16 (0.06–0.28)	0.10	0.01

BCLC: Breast Cancer Linkage Consortium.

cancer is carrying a *BRCA1* or *BRCA2* mutation. In this cohort, Ashkenazic Jewish status was not included in the predictive model, because the Ashkenazic Jewish group did not have a significantly different percentage of mutations compared with the non-Jewish group. These results suggest that a strong family history is a powerful predictor of the likelihood of carrying a mutation, regardless of ethnicity.[58] Researchers at Duke University[65,66] developed a model to evaluate the probability that a woman carries a mutation in *BRCA1* or *BRCA2*, based on her family history of breast and ovarian cancers. Using a Bayesian approach, the Duke researchers incorporate information about the families' possible genetic status, age-specific incidence of breast and ovarian cancers in carriers, and mutation prevalence in the population. These values can be changed to customize the model for subpopulations. For example, for Ashkenazic Jewish women, different allele frequency and age-specific penetrance are used in the calculation to obtain more accurate estimates for use in counseling.

Determining the probability that an individual is carrying a *BRCA1* or *BRCA2* mutation is only half of risk assessment. The other probability that must be determined is the likelihood of a mutation carrier developing cancer by a given age (i.e., age-specific penetrance). This is the point at which risk assessment becomes especially problematic, because all the factors that contribute to the development of cancer have not been identified. Not all individuals who carry mutations develop breast cancer or any other cancer. Expression is variable. For example, *BRCA2* mutation carriers may develop breast cancer, ovarian cancer, pancreatic cancer, fallopian tube cancer, or ocular melanoma. Even among families with founder mutations, there appear to be differences in age of onset of cancer and in the type of cancers that develop.[26,28,37,67] Expression and penetrance can vary from early onset bilateral breast cancer with ovarian cancer to late-onset breast cancer and from no other cancers in the family to additional cancers such as prostate, pancreatic, and other cancers. Therefore, it is not possible to assign mutation-specific risks. However, it is important to provide individuals with estimates of the likelihood of developing cancer.

The risk of developing breast or ovarian cancer when carrying a mutation varies in relation to the cohort studied (Table 4). The Breast Cancer Linkage Consortium (BCLC) risk estimates[68,69] are derived from families with several affected breast and or ovarian cancer cases. The estimates of Struewing et al.[70], which are for Ashkenazic Jews with any of three founder mutations, appear to be lower than the BCLC estimates. However, the estimates are not inconsistent, given that the confidence intervals overlap and both have similar risks (55%) by age 60. The existence of true differences could be explained by ascertainment- or mutation-specific differences. The Claus estimates[40] are for individuals in the general population who carry a susceptibility allele (q = 0.033). The risks for developing breast or ovarian cancer are high for mutation carriers, regardless of the variation in the estimates. They may be lower in those mutation carriers with little or no family history. The general population rates are also shown,[1] and they are relatively low. Estimates are that 1 in 8 women in the United States will develop breast cancer over the course of a lifetime, which includes the 5 to 10% of women carrying a high-penetrance gene predisposing to breast and/or ovarian cancers.

BRCA1 and *BRCA2* mutations are certainly important determinants of risk for breast and/or ovarian cancers, but they are not the only ones. Many women who have a family history of breast and/or ovarian cancer and do not have a *BRCA1* or *BRCA2* mutation may have a mutation in undiscovered genes. Moreover, some women may be *BRCA1* or *BRCA2* mutation carriers in the absence of a strong family history. This is especially true in women of Ashkenazic Jewish descent. For a subset of women, better predictions about

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their likelihood of developing breast and/or ovarian cancer at an early age can be made using *BRCA1* and *BRCA2* test results. However, even knowing mutation status does not always allow for valid risk estimates. Missense mutations are a good case in point, because the role of most of them is unknown.

CONCLUSION

One conclusion that can be drawn from this area of research is that there is ethnic variation in cancer risk that is probably the result of both genetic and epidemiological factors. Many genes have been isolated that are known to predispose humans to cancer. Founder mutations have been identified in many of these genes in different ethnic groups. Their further characterization is important because it will allow for more accurate risk assessment and more astute genetic counseling. However, the presence or absence of a founder mutation does not exclude the possibility of another mutation.

The risk of breast and ovarian cancers in mutation carriers is much higher than that in the general population, even given variable estimates depending on the population studied. Although estimates for risks of developing other cancers are not generally available, genetic counselors and physicians must be aware of the possibility of increased risks for other cancers as well.

Knowledge of which factors—genetic, environmental, or both—affect cancer development is essential for designing effective screening methods, providing information on ways to reduce cancer risk, and developing effective treatments once cancer develops. By studying the effect of a single, frequent mutation (founder mutation) in a well defined population, knowledge is gained that can be applied to larger, more heterogeneous populations. The founder mutations in *BRCA1* and *BRCA2* in Ashkenazic Jewish populations are the first to be examined in detail, and the data that are generated as a result of these studies are likely to provide information that will aid in the development of strategies for more successful prevention and treatment of breast and ovarian cancers.

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Ethnic Differences in Cancer Risk Resulting From Genetic Variation

Susan L. Neuhausen

Ethnic variation resulting from founder mutations has been identified in some cancer genes, so that a larger proportion of cancer in that ethnic group may be explained by one gene. However, founder mutations do not necessarily lead to higher overall cancer risk in that population.

Am J Hum Genet 1998 Oct;63(4):1224-7

Double heterozygotes for the Ashkenazi founder mutations in BRCA1 and BRCA2 genes.

Friedman E, Bar-Sade Bruchim R, Kruglikova A, Risel S, Levy-Lahad E, Halle D, Bar-On E, Gershoni-Baruch R, Dagan E, Kepten I, Peretz T, Lerer I, Wienberg N, Shushan A, Abeliovich AD

Publication Types:

- Letter

PMID: 9758598, UI: 98431808

Authors for ref. 54

ABSTRACTS

Schwann cells, and the adrenal medulla. Loss of *NF1* gene expression has been reported in Schwann cell tumors (neurofibrosarcomas) from patients with NF1 as well as malignant melanomas and neuroblastomas from patients without NF1. Previously, we demonstrated the lack of neurofibromin expression in six pheochromocytomas from patients with NF1, suggesting that neurofibromin loss is associated with the progression to neoplasia in pheochromocytomas in these patients. The lack of *NF1* gene expression in NF1 patient pheochromocytomas supports the notion that neurofibromin might be an essential regulator of cell growth in these cells. To determine whether *NF1* gene expression is similarly altered in pheochromocytomas from patients without NF1, twenty pheochromocytomas were examined for the presence of *NF1* RNA by reverse-transcribed PCR (RT-PCR). Lack of *NF1* gene expression was documented in four of these twenty tumors (20%) which corresponds to previously reported numbers for malignant melanomas and neuroblastomas in non-NF1 patients. Of these twenty pheochromocytomas, one of four sporadic tumors, one of ten tumors from patients with MEN2A, one of four tumors from patients with MEN2B, and one of two tumors from patients with von Hippel-Lindau syndrome demonstrated loss of *NF1* gene expression. In all cases, the quality and quantity of tumor RNA was determined by RT-PCR amplification using primers which amplify cyclophilin RNA. We previously demonstrated that these tumors do not harbor activating mutations of the N-ras, K-ras or H-ras proto-oncogenes. These results suggest that loss of *NF1* gene expression is frequently associated with the progression to neoplasia in tumors derived from adrenal medullary tissue in patients without clinical manifestations of neurofibromatosis and supports the notion that neurofibromin is a tumor suppressor gene product involved in the pathogenesis of a wide variety of tumor types.

306

A 45-year followup study of breast and other cancers in kindred 107 and linkage analysis of candidate loci. D.E. Goldgar[1], D.F. Easton[1,2], S.L. Neuhausen[1], J.H. Ward[1], L.A. Cannon-Albright[1], M.H. Skolnick.[1] [1]University of Utah, Salt Lake City. [2]CRC Section of Epidemiology, Institute of Cancer Research, Sutton, UK

One of the earliest large kindreds with inherited susceptibility to breast cancer was reported by Gardner and Stephens in 1950. This family, denoted K107, was ascertained in 1947 by a genetics student with two great aunts who died of breast cancer in their 40's. Subsequent clinical and genealogical follow-up identified 7 additional cases of early-onset breast cancer. The family was updated several times, most notably in 1980 (Gardner and Bishop, 1980). For the present study K107 was recently reinvestigated and over 75 blood samples gathered for genotyping. The kindred now contains 38 cases of female breast cancer, 3 cases of male breast cancer, and 6 cases of ovarian cancer, 18 of which have been identified since the 1980 report. Examination of the obligate carriers demonstrates that the gene responsible for the breast and ovarian cancer in K107 is highly penetrant. Other cancers appear to be associated with expression of this gene, most notably prostate cancer, melanoma, and uterine cancer. Linkage to the BRCA1 region in K107 was excluded based upon the analysis of genotypings at four loci covering the BRCA1 gene on chromosome 17q has been excluded in this family, using four highly polymorphic markers in the BRCA1 region (multipoint LOD score -3.27). Eight other candidate breast cancer susceptibility genes and candidate regions, including p53 and ESR have also been tested for linkage and excluded. Studies to formally re-estimate penetrance and test for excesses of all cancer sites and a genomic search in this family are in progress.

308

Elevated levels of somatic mutation at the glycophorin A locus in cancer patients: Relevance for incidence of secondary cancer. S.G. Grant and W.L. Bigbee. Center for Environmental and Occupational Health and Toxicology, University of Pittsburgh, PA.

The glycophorin A (*GPA*)-based human *in vivo* somatic mutation assay was used to survey a large number of newly diagnosed cancer patients presenting with a variety of solid tumors. This cancer patient population was sampled pre-therapy and frequencies of two types of variant cells determined: allele loss segregants arising by mutation, deletion, chromosome loss or gene inactivation, and allele loss and duplication segregants arising by chromosome missegregation, mitotic recombination and possibly gene conversion. When compared with matched controls, the cancer patient population exhibited significantly elevated frequencies of both types of segregants. Cancer patients undergoing genotoxic therapy with chemicals and/or ionizing radiation were then examined, yielding

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Analysis of mutations in brain, thymus and testis from lacI containing transgenic mice: do the spontaneous mutation frequencies and patterns change with age? V. L. Buettner[1], H. Nishino[1,2], J. Haavik[1], and S. S. Sommer[1]. Mayo Clinic/Mayo Foundation, Departments of Biochemistry and Molecular Biology[1] and Neurology[2], Rochester, MN.

Recently developed transgenic mouse detection systems offer a powerful tool for analysis of spontaneous mutations *in vivo*. Big Blue™ mice carry chromosomally integrated lambda bacteriophage shuttle vectors (LIZ) containing the *E.coli* lacI gene as a target for mutagenesis. Lambda shuttle vectors rescued by *in vitro* packaging can be screened for mutations in the lacI gene by using α complementation and X-gal as a substrate. Herein, Big Blue™ mice were used to examine the rate and pattern of spontaneous mutation *in vivo* with respect to age. Four mice each were sacrificed at 3 and 10 months. Three tissues were examined: brain, thymus and testis. Nine million plaques were screened from all three tissues and a total of 287 blue (mutant) plaques were harvested and replated. The mutant frequencies in the 3 and 10-month-old mice were not statistically different ($2.9x10^{-5}$ and $2.8x10^{-5}$ in the brain, $2.8x10^{-5}$ and $4.1x10^{-5}$ in the thymus, and $3.9x10^{-5}$ and $3.6x10^{-5}$ in the testis). The entire lacI gene (1.2 kb) was sequenced from 69 blue plaques harvested from brain. A single mutation was found in the lacI gene from 68 of the plaques. When recurrent mutations from the same mouse were excluded, 63 independent mutations were found in total. There were no statistically significant differences in the mutational spectra in the brain between 3 and 10 month old mice ($p=0.4$; Fisher's Exact Test). This suggests that a 3-fold increase in age does not affect the mutational frequency or pattern in the brain. Analysis of the age-dependent pattern of mutation in thymus and testis is in progress.

Terminal deletion and multiple breakpoint cluster in the chromosome 3p region of sporadic and hereditary renal cell carcinomas. P. Bugert, M. Wilhelm, C. Kenck, G. Staehler and G. Kovacs. Clinical Research Group, Molecular Oncology, Dept. Urology, Ruprecht-Karls-Univ., Heidelberg, Germany.

The development of nonpapillary renal cell carcinomas (npRCC) is associated with the loss of 3p sequences. The most distal breakpoints have been found at the boundary between the chromosomal bands 3p14.1 and p13. Thus, the RCC gene is thought to be located in the 3p region. Recently, the VHL gene has been cloned from the chromosome 3p25-26 region. The gene is considered to be the RCC gene but only about 50 % of analyzed tumors with the deletion of one allel showed a mutation of the other VHL allel. So, another tumor supressor gene is involved in npRCC development and should be located more proximal to the VHL locus, presumably proximal to 3p14.2. The microsatellite analysis was used to determine the RCC gene locus in sporadic and hereditary tumors including those of which are associated with constitutional balanced translocations 3;6 and 3;8. All tumors analyzed showed a terminal deletion distal of the D3S1300 locus at 3p14.2 and no interstitial deletion was found. In about 30% of the tumors a common breakpoint was found between the D3S1251 and the D3S1101 locus with about 1 cM genetic distance at 3p11.2. Other breakpoints clustered between the D3S1663 locus (p11.2) and the D3S1300 (p14.2) locus including the constitutional breakpoints in the 3;6 and 3;8 translocation families with renal cell carcinomas.

319

Frequency and spectrum of inherited mutations in BRCA1 from a population-based study. L.M. Butler 1, B. Newman 2, L.S. Friedman 1, E.A. Ostermeyer 1, J.E. Morrow 1, P. Dowd 1, P.G. Moorman 2, R. Millikan 2, M-C. King 1. 1 Univ. California, Berkeley; 2 Univ. North Carolina, Chapel Hill.

Heretofore, highly penetrant alleles of BRCA1 have been identified in families selected for high risk of breast or ovarian cancer. But are there BRCA1 mutations that confer lower risk than those found in high-risk families? What is the frequency of inherited BRCA1 mutations among breast cancer patients from the general population? Does the frequency or spectrum of inherited BRCA1 mutations vary by age at diagnosis or by race? Do environmental factors influence penetrance of some BRCA1 mutations? What is the role of genes such as the androgen receptor (AR), at which polymorphism may have a moderate influence on breast cancer risk? To address these questions, a population-based series of 900 breast cancer patients was selected from 21 counties of central and eastern North Carolina. The sample was designed to include equal numbers of African-American and Caucasian patients, and equal numbers of women diagnosed younger than 50 years and at age 50 or older. Family histories and information on environmental risk factors was obtained by interview. Age and race-matched controls were also sampled. Based on preliminary data from 5% of BRCA1 sequences, three distinct mutations have been found in four patients. These early data suggest that: (1) the BRCA1 mutation spectrum will vary considerably across populations, with founder effects important within any one group; (2) mutations leading to splicing errors may be relatively more frequent in the general population of patients; (3) distinguishing rare disease-related mutations vs rare polymorphisms requires a large and appropriately matched series of controls; (4) BRCA1 mutations occur among probands with both younger and older ages at onset and among both races. Penetrance of these mutations will be evaluated by testing both affected and unaffected relatives of probands. The polymorphic polyglutamine repeat in the N-terminal domain (CAG repeat in exon 1) of the androgen receptor (AR) will be evaluated in the same series to determine whether inherited variation in this functionally significant region contributes to breast cancer risk in the general population.

320

Increased risk of stomach cancer in BRCA2 carriers. L.A. Cannon-Albright[1], S. Neuhausen[1], M. Higbee[1], M. Skolnick[1,2]. [1]University of Utah School of Medicine, [2] Myriad Genetics, Inc., Salt Lake City.

Increased risks for ovarian cancer as well as colon and prostate cancers are recognized for BRCA1 mutation carriers. It is not yet clear whether BRCA2 increases risks for cancers other than breast; however, in a kindred with evidence of BRCA2 linkage, an excess risk of stomach cancer has been noted. Kindred K4328 descends to 310 individuals in six generations. The kindred was ascertained for an excess of prostate cancer; 8 cases were observed and 2.42 were expected ($p<0.01$). Examination of the descendants revealed an excess of breast cancer (6 obs., 2.43 exp., $p=0.03$), an excess of stomach cancer (6 obs., 0.30 exp., $p<.001$), and a single early onset ovarian case (0.40 exp., $p<0.3$). Because 4 of the breast cancer cases were diagnosed before age 50 the family was typed for BRCA1 and BRCA2 linkage. No linkage to BRCA1 was observed for all cancers, nor for the breast cancer cases alone. The four breast cancer cases which could be typed share an 11 marker haplotype on chromosome 13q from D13S290 to D13S267. In addition, the 4 stomach cancers which could be typed also share this 11 marker haplotype. Two stomach cancers at age 47 and 62 were untyped. Other cancers observed in BRCA2 susceptibility haplotype carriers include pancreas at 71, prostate at 86, lung at 49, and cervical cancer at 25. Allele loss in gastric and lung tumors at 13q12-22 has been reported. Only one of the 8 prostate cancer cases carries the BRCA2 susceptibility haplotype. Although this family differs from the typical high penetrance BRCA2 kindreds, it may represent a lower penetrant variant which could be responsible for a portion of breast cancer and stomach cancer which is less commonly recognized as familial.

Poster Presentations

321

Loss of heterozygosity at TSC1 and TSC2 loci in TSC-associated and sporadic hamartomas. C. Carbonara(1), L.Longa(1), F.Grosso(1), G.Mazzucco, C.Borrone, M.L.Garrè, M.Brisigotti, A.Giannotti, P.Falzoni, G.Monga, G.Garini, M.Gabrielli, P.Riegler, C.Danesino, M.Ruggieri, G.Magro, G.Filippi, A.Scabar, F.Bonetti, M.Pea, G.Martignoni, N.Migone(1). (1)CNR Centro Immunogenetica ed Oncologia Sperimentale and Dipartimento di Genetica, Biologia e Chimica Medica, Università di Torino; TS Italian Collaborative Group, Italy.

Loss of heterozygosity (LOH) at TSC1 and TSC2 loci, as well as at seven tumor suppressor containing regions (p53, NF1, NF2, BRCA1, APC, VHL and MLM) has been investigated in 20 TSC-associated hamartomas and in 17 patients carrying sporadic angiomyolipomas (15 renal and 2 hepatic). The 20 TSC-associated hamartomas included 8 renal angiomyolipomas, 8 giant cell astrocytomas, 1 cortical tuber and 3 rabdomyomas, derived from a total of 18 patients (14 sporadic and 4 familial). In the TSC-associated lesions, TSC2 LOH was observed in 5 angiomyolipomas and in 4 astrocytomas; TSC1 LOH was found in only one astrocytoma from a TSC1-linked family (Carbonara C. et al. Hum. Mol. Genet. 3:1829, 1994). Overall a significant preponderance of TSC2 LOH was documented. A series of possible explanations were considered: a) a TSC2 gene higher mutation rate; b) the likelihood that the TSC1 defects are mostly microdeletions or point mutations, undetectable by the LOH method; c) hamartomas bearing TSC2 mutations might more frequently necessitate surgical excision. No loss of heterozygosity has been observed at the others tumor suppressor containing regions.

LOH spanning different regions has been observed in two samples (astrocytoma and renal angiomyolipoma) from the same patient. Thus supporting the multifocal origin of the second mutation.

Among the sporadic angiomyolipomas, TSC2 loss of heterozygosity has been observed in 2/11 informative renal angiomyiolipomas and in 1/2 hepatic angiomyolipomas. This observation is consistent with a role of TSC2 in the development of sporadic hamartomas.

322

Molecular Characterization of der(1)t(1;2)(p36;q31) in non-Hodgkin lymphomas. K. Carlson, S. Bajalica and M. Nordenskjold. Department of Clinical Genetics. Karolinska Hospital. Stockholm. Sweden.

Chromosomal rearrangements are frequently associated with neoplasias. Translocations involving 1p36 are found in 17% of all NHL while 80-90% of all folicullar non-Hodgkin lymphomas (NHL) are found to contain a 14;18 translocation. Using fluorescence in situ hybridization (FISH) with biotin labeled chromosome-specific libraries (chromosome painting) on metaphases from short-term cultures from follicular non-Hodgkin lymphomas, we identified three cases with der(1)t(1:2)(p36;q31). Furthermore, two normal chromosome 2 homologues were found in addition to the der(1)t(1;2) indicating a partial trisomy of the distal part of chromosome 2 in the three cases. In one of the cases, the der(1)t(1,2) appeared as the sole aberration. By PCR analysis we were able to show that none of these tumors had the 14;18 translocation despite their follicular growth pattern. We performed subtraction hybridization with both chromosome 1 and chromosome 2 specific libraries and a genomic phage library derived from one of the der(1)t(1,2) patients. A genomic clone containing an open reading frame which maps to chromosome 2 was identifed. Continued analysis of this and other clones from our subtraction screen may give us more insight into the genetic material involved in lymphoma-associated translocations. In addition, two genes, the leukemia-associated phosphoprotein, p18 and a cAMP-dependent protein kinase are known to map to 1p36. These will be regarded as potential genes involved in the development of follicular lymphomas which do not involve the t(14;18).

conventional indicators of poor prognosis, are at high risk of early recurrence and death. The dramatic findings in this study may reflect a peculiarity of the sampled population. Alternatively, the results may well reflect the importance of utilizing methodology that detects most, if not all, of the mutations present in BC (see above).

20

Identification of candidate exons from the BRCA2 region using exon amplification. F.J. Couch[1], K.F. Hoskins[1], Y. Peng[1], M.L. DeShano[1], S.V. Tavtigian[2], J. Weaver-Feldhaus[2], S. Neuhausen[3], M.F. Stringfellow[2], E.T. Janecki[2], K.J. Abel[4], and B.L. Weber[1]. [1]University of Pennsylvania Medical Center, Philadelphia, Pennsylvania; [2]Myriad Genetics, Salt Lake City, Utah; [3]University of Utah, Salt Lake City Utah; [4]University of Michigan, Ann Arbor, Michigan.

Family history of breast cancer has been identified as a major risk factor in the development of early onset breast cancer. An autosomal dominant susceptibility gene (BRCA2) has recently been localized to chromosome 13q12-13 by linkage analysis. The gene is known to reside in a 6 cM region between the markers D13S267 and D13S289.

A 2 Mb BAC/P1/PAC contig was generated using STSs derived from the ends of YACs in the candidate region. Exon amplification was performed using a subset of the BACs and P1s in order to isolate a large number of gene sequences from the BRCA2 candidate region. Six pools of genomic clones were assembled, each containing approximately 300 kb of DNA. Genomic clones were digested with PstI or BamHI + BglII and ligated into the PstI or BamHI sites of the pSPL3 splicing vector. The exon amplification technique was performed and the end products were cloned in the pAMP1 plasmid from the Uracil DNA Glycosylase cloning system (BRL). Approximately 2000 clones were picked, propagated in 96 well plates, stamped onto filters, and analyzed for the presence of vector and repeat sequences by hybridization. Each clone insert was PCR amplified and tested for redundancy, localization, and human specificity by hybridization to grids of exons, dot blots of the parent genomic clones, and southern blots of human, E. coli and yeast DNAs respectively.

To date 50 unique candidate exons have been identifed and have been positioned on the contig in the form of a transcription map of the BRCA2 candidate region. Each exon is used for cDNA library screening with subsequent cDNA clones being used for Northern blot and mutation analysis. Sequence analysis of these exons failed to identify any previously known sequences by database alignment. These exons and cDNAs will greatly facilitate the cloning of the BRCA2 gene by rapidly identifying many candidate genes within the BRCA2 region.

Slide Session 22: Molecular

21

Endothelin receptor B gene mutation in a consanguineous family with Waardenburg-Hirschsprung's disease ((T. Attié, M. Till*, J. Amiel, P. Edery, A. Pelet, A. Munnich, S. Lyonnet)) Service de Génétique Médicale et Unité INSERM U-393, Hôpital des Enfants-Malades, 75743 Paris, France; * Service de Pédiatrie et de Génétique, Hôpital Debrousse, Lyon, France.

Both Hirschsprung's disease (HSCR) and Waardenburg

, and Children's Med. Ctr. Stoney

gative, clonal myeloproliferative
in a multipotent hematopoietic
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of an unidentified predisposition
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¹University of Groningen, The Netherlands, and University ...
Denver, CO.

Human chromosome 3 has been implicated in a number of human cancers. One locus has been defined at 3p21.3 which shows homozygous deletion in small cell lung cancer (SCLC). A fragment of human DNA overlapping the deleted region can suppress tumor formation by mouse A9 cells. The region in common spans approximately 400 Kb. Using AluPCR fragments from the somatic cell hybrid containing the fragment of human DNA on the mouse background, we have designed primers for the inter Alu sequences in the region. The markers D3S1573 and D3S1235 as well as the GNAI2 gene are encompassed in the common region. Using the end sequences from our entry clones a P1 contig was constructed. Each of the P1 clones were cotransfected with ptkhyg plasmid into A9 cells and resistant clones were screened for chromosome 3 markers. Each transfectant was then tested for their growth in nude mice. One of the P1 clones (P1 294) suppressed tumor formation in A9 cells. This P1 clone which has 74 Kb of human DNA was used to isolate cDNA clones using exon trapping, hybridization selection, and identification of CpG islands. This region of the genome appears to be quite gene dense. One gene was found which is homologous to the semaphorin gene family and is 50% homologous to chicken collapsin. As loss of heterozygosity is nearly 100% in this region in small cell lung cancer, we expect to find mutations in the SCLC tumor samples and cell lines when we isolate the bona fide tumor suppressor gene. However, mutations were not found in the semaphorin homolog in lung cancer samples. The α transducin (GNAT1) gene is also located on this P1 clone; however, GNAT1 is retinal specific and not expressed in lung. We have found at least 4 other genes on P1 294, three of which are expressed in normal lung. These transcripts do not have homology to known genes in GenBank. We are screening lung cancer samples for mutations in these genes. (Supported by CA56626).

Poster Presentations

...somes. J. A. Neidich, C. D. Bangs, C. L.
Univ. School of Medicine, Stanford, CA.
owever, it is the most common pediatric
oblastomas have identified partial or full
on events, but only 20 cases have been
on of chromosome 2 has been q23-q35.
reported karyotype aberrations seen in
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d what was interpreted as a translocation
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394

A physical map of the BRCA2 region constructed in P1s, BACs, and PACs and assessment of loss of heterozygosity (LOH) in familial BRCA2 tumors. S.L. Neuhausen[1], C.J. Marshall[1], J. Swensen[1], Y. Miki[1], L. Cannon-Albright[1], J. Weaver-Feldhaus[2], M. Stringfellow[2], E.T. Janecki[2], D.E. Goldgar[1], M.H. Skolnick[1,2], A. Kamb[2] and S.V. Tavtigian[2].
[1]University of Utah and [2]Myriad Genetics, Salt Lake City, Utah.

A second familial breast cancer gene, BRCA2, has been localized to 13q12-13 by linkage analysis (Wooster et al., 1994, Science 265: 2088-2090). Using published markers in the region, we constructed a YAC contig spanning the interval from D13S289 to D13S267. STSs generated from YAC ends and the published markers were used to isolate initial P1s, PACs, and BACs in the region. Chromosomal walks from the ends of those clones allowed us to complete a P1/PAC/BAC contig spanning our genetically defined region of ~ 2 Mb. Polymorphic short tandem repeat markers (STRs), isolated from the genomic clones, were used to refine the BRCA2 region.

LOH was examined in breast and ovarian tumors from both carriers and sporadic cases of five BRCA2-linked kindreds using eight BRCA2-linked polymorphic STRs and one STR near retinoblastoma (RB). Two of the three sporadic cases examined did not show LOH, whereas one exhibited LOH of the entire region including RB. Of the seven tumors from carriers, two exhibited no LOH, two had LOH of the entire region including RB, and three had interstitial loss. In all cases where LOH was observed, the wild type allele was lost. These results confirm the findings of Collins et al. (1995, Oncogene 10: 1673-1675), and provide additional evidence that BRCA2 is a tumor suppressor gene.

01 PSYCHOLOGICAL ASPECTS OF CANCER GENETIC CONSULTATION

Julian C[1], Eisinger F[1], Chabal F[1], Aurran Y.[1], Noguès C[2], Bignon Y-J[3], Maugard C[4], Machelard M[5], Vennin P[6], Sobol H[1].
1 INSERM U379-Inst. Paoli-Calmettes, Marseille, France 2 Ctre R.Huguenin, St-Cloud, 3. Ctre J. Perrin, Clermont-Ferrand, 4 Ctre R. Gauducheau, Nantes, 5. Ctre. Claudius Regaud, Toulouse, 6.Ctre Oscar Lambret, Lille.

The existence of family cancer clinics has been reported in the literature from the early 1990's. These clinics were in France first introduced in 1987 and progressively extended in 1995 to more than 30 different cities all over the country. The aim of this study was to investigate the impact of cancer genetic consultations on clients' perception of cancer risk and on their feelings of anxiety. Among the healthy consultees attending from January 94 to January 95, any of six French clinics located in different cities, 89.9% (N=213) answered a pre and a post-consultation questionnaire. After the consultation, the oncogeneticists filled in a standardized form giving their risk assessment. The average age was 43.5 years (SD=12.6) and the first reason to consult was in 77.4% for breast cancer in the family. 90% of the sample had at least one first degree relative with cancer but 67% were assessed, by the oncogeneticist, to have a cancer risk running in their family. On average, the subjects felt less anxiety (Spielberger's State Trait Anxiety Inventory) after than before the consultation ($p<0.001$). The certainty around the risk existence and the magnitude of the risk were the main factors determining accurate perception of an increased family risk after the consultation ($p<0.05$). Among the healthy 'at risk' patients (n=142), a more accurate perception of their personal cancer risk was found, after multivariate adjustment by logistic regression, to be strongly associated with perception of an increased family risk ($p<0.001$), with consultation at the family's request ($p=0.003$), with a higher educational level ($p=0.012$) and with a change in the medical surveillance carried out by the oncogeneticist ($p=0.023$) ; those who rightly perceived an increased risk were also less reassured by the consultation than those who perceived the risk inaccurately ($p= 0.008$). The results of this study are discussed in terms of their implications for the medical information that should be given at the first cancer genetic consultations.

02 PROGNOSIS AND ACUTE RESPONSE TO RADIATION THERAPY IN BREAST CANCER PATIENTS WITH BRCA1 AND BRCA2 MUTATIONS.

Gaffney, DK*, Lewis, CM, Brohet, R, Cannon-Albright, L, Holden, J, Buys, S, Ward, J, Neuhausen,S, Shattuck-Eidens, D, Avizonis, V,et al. University of Utah, Salt Lake City, UT, USA

Purpose To evaluate overall survival in breast cancer patients with genotypically characterized mutations in BRCA1 or BRCA2, describe presenting stage, review histologic findings, and evaluate acute response to radiotherapy.

Methods A retrospective study was performed evaluating breast cancer patients with known mutations of BRCA1 or BRCA2. Patients from 12 different pedigrees were cross referenced with the Utah Cancer Registry (UCR), histologic findings were verified, and radiotherapy records were reviewed for acute response to treatment.

Results Forty-six breast cancer patients with known BRCA1 or BRCA2 mutations were identified. Twenty-seven breast cancer patients with BRCA1 mutations were found to have 31 breast cancers (4 had bilateral, metachronous lesions), and 19 breast cancer patients with BRCA2 mutations were found to have 20 breast cancers (1 had bilateral, metachronous disease). The median age at diagnosis was 49 (range 21-77) and 42 years (range 23-83), respectively for BRCA1 and BRCA2 patients. Unusual histologic types of breast cancers were represented with 6% medullary and 6% lobular carcinomas. Complete staging was possible for 61% (31/51) of cancers. Stages I, II, III, and IV represented 26%, 61%, 6%, and 6%, respectively. The surgical procedure was mastectomy in 88% (45/51) of lesions. Breast conservation therapy (conservative surgery and radiation) was utilized in 12% (6/51) of neoplasms, and one patient experienced a local failure 3 years after diagnosis. The most severe radiation reaction was moist desquamation which was self-limiting and developed in 26% (5/19) of irradiated patients. Mean follow up was 9.8 and 7.5 years for BRCA1 and BRCA2 cancers, respectively. Kaplan Meier survival analysis demonstrated:

	N	%5 yr survival	%10 yr survival
BRCA1	31	80	56
BRCA2	20	75	50
UCR	18565	68	50

No statistically significant differences were evident between the three groups at 5 or 10 years by chi square analysis.

Conclusions Breast cancer patients harboring BRCA1 or BRCA2 mutations compared with sporadic breast cancer patients present at a similar stage, display a normal acute reaction to radiotherapy, and have a similar prognosis despite their younger age at presentation.

to monitor residual disease, evidenced by detection of abnormal clones observed at presentation, and the ascertainment of successful ablation of neoplastic host cells, as evidenced by the percentage of male (host) cells versus female (donor) cells determined by interphase FISH. Mitotic cells collected from cultures using lipopolysaccharide (LPS) or lymphocyte conditioned medium (LCM) with Epstein Barr virus (EBV) supernatant were consistently cytogenetically normal, with a 46,XX karyotype. Cytogenetic analyses from cultures stimulated with either pokeweed mitogen, *Staphylococcus aureus* protein A (SPA), phorbol 12-myristate 13-acetate (PMA) plus tumor necrosis factor (TNF) or interleukin-2 revealed a chimeric state with persistence of a small population of aberrant male cells, corroborated by FISH interphase studies. FISH assays of interphase nuclei from LCM/EBV, SPA or PMA/TNF cultures revealed significantly different percentages of Y signals. One comparative FISH study of a bone marrow specimen detected Y signals in 2.0% of cells from the uncultured marrow, 5.6% in LCM/EBV cells, and 74.9% in SPA cells. A subsequent FISH study again gave discordant results with a 1.7% Y signals in LCM/EBV cells versus 15.3% in SPA cells.

An intriguing possibility is raised that choice of mitogen is a major factor in detection of aberrations in CLL, based on variable sensitivity of neoplastic cells to specific mitogens. Sequential monitoring may result in identification of prognostic subgroups defined by chromosomal abnormalities and mitogen-sensitivity

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404 Poster Symposium–Session 32

Frequent occurrence of specific germline mutations of BRCA1 and BRCA2 in Ashkenazi Jewish women with breast cancer.

B. Haas, I. Forsyth, D. Hochhauser, S. Neuhausen, T. Gilewski, H. Hampel, K. Brown, P. Borgen, L. Norton, K. Offit. Memorial Sloan-Kettering Cancer Center, New York, N.Y. and University of Utah, Salt Lake City, Utah

The lifetime risk of breast cancer may approach 80-90% in women who have germline mutations of either of two genes, *BRCA1* or *BRCA2*. A single *BRCA1* mutation, 185delAG, has been noted in 0.9% of the Ashkenazi population. We investigated the frequency of the 185delAG, 5382insC *BRCA1* mutations and 6174delT *BRCA2* frameshift mutations in 80 women with early-onset breast cancer (before 42) and 27 additional Jewish probands with a positive family history of breast or ovarian cancer in which the proband was diagnosed with breast cancer between the ages of 42 and 50. Twenty-five mutations (16 delAG, 6 delT,3 insC) were identified in the first group of 80 cases and 11 mutations (8 delAG, 2 delT, 1 insC) were identified in the second group of 27 cases. Of the 36 heterozygotes, all had a family history of breast or ovarian cancer, except for 2 individuals with the 6174delT mutation of *BRCA2*. Of 21 Jewish individuals with breast cancer and a family history of ovarian cancer, 15 (71%) had one of these three mutations. These results suggest that two recurrent mutations of *BRCA1* and a mutation of *BRCA2* may account for more than a quarter of early-onset breast cancer cases, and almost three quarters of breast cancer cases with a family history of ovarian cancer in the Jewish population. Analysis of pathologic and clinical features of these cases is in progress.

405

Chimeric EGF/RET tyrosi
regulation compared to EG

and L. Pike[3,1]. Div. Hum. M
Washington Univ. Sch. Med., S

The RET gene encodes a recept
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Diagnostic Laboratory, Department of Genetics, University of Pennsyl
Philadelphia, PA 19104.

Mutation analysis of cancer susceptibility genes has opened up a new era in
genetics. However, clinically feasible testing is technologically challenging
genes, such as BRCA1 and BRCA2, are usually large and multi-exon with
mutations. With a view to offering rapid and reliable mutation analysis
patients with a positive family history of breast and ovarian cancer syndrome, we
automated our conformation sensitive gel electrophoresis (CSGE) mutation
assay. The BRCA2 gene is amplified in 54 separate fragments covering all 27
exons and the exon-intron boundaries from genomic DNA using PCR
which one is fluorescently labelled with one of the three dyes (FAM-blue, HEX-
and TET-green). After PCR, PCR products of three different color are analyzed
single lane of the ABI 377 machine allowing analysis of 108 PCR products in
hour run. This translates into preliminary screening of the entire coding sequence
BRCA2 gene for 4 patients, or the BRCA1 gene for 7 patients, in two days
analysis time is taken into consideration. The gel image is analyzed using the
analysis software, Gene Scan (ABI, CA) and directly exported to specific
each individual. The advantages of this technology are the unparalleled reproducib
and resolving power of the scanning method. Multiplexing of different PCR
now been attempted. Only the aberrantly-migrating heteroduplex bands are
To date, we have completed mutation analysis of the BRCA2 gene of 26
affected with breast and or ovarian cancer and a positive family history, but negative
BRCA1 mutations. Numerous polymorphisms have been detected. Five mutations
been confirmed in the BRCA2 coding sequence including two 6174 delT mutations
exon 11 of BRCA2. None of these families had any other male member affected
breast cancer nor was there any distinctive cancer in any of these families to
which BRCA gene should be analyzed. (Supported by a grant from NIH,
1R21CA66179 and use of an ABI 377 machine from PE ABD, CA)

167

The Carrier Frequency of the BRCA2 6174delT Mutation Among Ashkenazi Jewish Individuals is Approximately 1%. C. Oddoux[1], J. P. Struewing[2], C. M. Clayton[1], L. C. Brody[2], S. Neuhausen[3], M. Kaback[4], D. Goldgar[5], H. Ostrer[1], K. Offit[6]. [1]New York University Medical Center, New York, NY, [2]National Institutes of Health, Bethesda, MD, [3]University of Utah, Salt Lake City, UT, [4]University of California, San Diego, CA, [5]International Agency for Research on Cancer, Lyon, France, [6]Memorial Sloan-Kettering Cancer Center, New York, NY

Certain germline mutations in either the *BRCA1* or *the BRCA2* gene confer a lifetime risk of developing breast cancer that may approach 90%. The 185delAG *BRCA1* mutation was found in 20% and the 6174delT *BRCA2* mutation in 8% of Ashkenazi Jewish women with early-onset breast cancer. The 185delAG mutation was observed in 0.9% of 858 Ashkenazi Jews unselected for a personal or family history of cancer. Assuming comparable age-specific penetrances, a frequency of 0.3% was estimated for the 6174delT *BRCA2* mutation . To test this hypothesis, we performed a population survey of 1255 Jewish individuals. In two independent groups, ascertained from heterozygote detection programs for other conditions, a prevalence of approximately 1% (CI 0.6 - 1.5) was observed of 6174delT. From these findings, the relative risk of developing breast cancer by age 42 was determined to be 9.3 (C.I. 2.5 - 22.5) for 6174delT, compared to 31 (C.I. 11 - 77) for 185delAG. Although these findings may be explained by chance variation, the more likely explanation is a difference in age-specific penetrance for the two mutations. These results indicate that one in 50 Ashkenazi Jewish individuals harbor specific germline mutations in *BRCA1* or *BRCA2* and that genetic counseling for these individuals must be tailored to reflect the different risks associated with the two mutations.

mutation in Jewish population
T. Iwata[1], B.C. Lanpher[1], J.P.
ational Center for Human Genome
niv., Washington D.C.
(BRCA1) is associated with high
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169

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a, A. A. Boyd, C. S. Richards,
ge of Medicine, Houston, Texas.
RCA2 gene on 13q12-13 are the
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BRCA1 Mutations in African-American Women J. F. Arena[1], S. Smith[1], M. Plewinska[1], L. Gayol[1], E. Perera[1], P. Murphy[2], and H. Lubs[1]. [1]University of Miami School of Medicine, Miami Florida. [2]OncorMed Inc., Gaithersburg, Maryland.

Breast cancer in African-Americans is associated with a poorer prognosis than in Caucasians. African-American women tend to present at an earlier age with larger tumors and a more advanced stage disease. Studies designed to detect a possible

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PATHOGENESIS OF BRCA1- AND BRCA2-RELATED BREAST CANCER

David K. Gaffney[1], Beverly Lynch[2], Joseph Holden[2], Margaret Lundquist[1], and Susan Neuhausen[3]
Departments of Radiation Oncology[1], Pathology[2], and Genetic Epidemiology[3]

Purpose: Previous reports have shown that BRCA1-related breast cancers are higher grade than sporadic breast cancers, yet survival is comparable or improved. Fewer data exist regarding BRCA-2 related breast cancer. In this study we examine molecular markers to discern the pathogenesis of hereditary breast cancer.

Materials and Methods: Tissue blocks were obtained on 33 BRCA1-related breast cancers, 18 BRCA2-related breast cancers, and 23 sporadic controls. The tumors were graded, flow cytometry was done, and immunohistochemistry was performed for ER, PR, p53, Mib-1, Topoisomerase II, Her2/neu, and factor VIII. The slides were read in a blinded fashion by a pathologist. ER, PR, and Her2/neu were reported as positive or negative, and p53, Mib-1, and topoisomerase II were reported as an index or the percentage of positive cells per multiple high powered fields. Factor VIII was scored as the number of microvessels per mm^2.

Results: The median age at presentation for the BRCA1 group (44 years) and BRCA2 group (45 years) was significantly younger compared with sporadic controls (64 years, p=0.009). There was no difference in tumor size between groups. The BRCA1 group had a decreased frequency of ER and PR positivity versus controls. The increased proliferative nature of BRCA1 tumors was demonstrated by a higher frequency of grade 3 (p=0.008), and increased topoisomerase II (p=0.001) and Mib-1 (p=0.001) staining versus controls; however, there was not an increase in percent S phase. Both BRCA1 and BRCA2 groups showed an increase in p53 expression (p=0.013 and p=0.007, respectively). Only one BRCA1 and one BRCA2 tumor showed Her2/neu staining. Microvessel density as determined by factor VIII staining was reduced in the BRCA2 group (p=0.02) and slightly reduced in the BRCA1 group (p=0.073) versus the control group.

Conclusions: BRCA1 and BRCA2-related breast cancers present at a similar size and over a decade earlier compared with sporadic breast cancers. BRCA1 cancers are higher grade and have a high proliferative rate as determined by increased expression of Mib-1 and topoisomerase II.
The increase in p53 expression and decrease in microvessel density may suggest cell cycle dysregulation in BRCA1- and BRCA2-related breast cancer.

...otein interactions. *A. Aronheim[4], R. Baer[3].* 1) ...enter, Dallas, TX, USA; 2) ...er, Dallas, TX, USA; 3) ...ter, Dallas, TX, USA; 4) Galim, Haifa, Israel.
...al RING domain and two ...sense mutations in either ...n pattern and subcellular ... performed a two-hybrid ...ed that it associates with ...T motifs. During progres... clear dots. Like BRCA1, ...umors, but are occasion... been found in more than ...ents of cell cycle check... BRCA1 is a potent reg... activity. To identify pro... Sos-recruitment system ...e a transcription readout. ...d fourteen independent ...in mammalian cells, only BRCA1. This was a pre... identified on the basis of ...servation reinforces the ...sts that BRCA1 controls ...scriptional repression.

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...se individuals to breast ...of BRCA1 – associated ...ne population studied. ...enes, play a role in the ...at chromosomes 4 and ...pment of breast cancer ...bridization in BRCA1 – ...loss (86%), followed by ...ing loci, we performed ...ers spanning chromo... either germline BRCA1

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247

Breast cancer risk in BRCA1/BRCA2 carriers is modified by the CYP1A1 gene. *J-S. Brunet[1], D. Vesprini[1], J. Abrahamson[1], S. Neuhausen[2], S. A. Narod[1].* 1) Centre for Research in Women's Health, Women's College Hospital, Toronto, On, Canada; 2) Genetic Epidemiology, University of Utah, Salt Lake, UTAH, United States.

The risk of breast cancer in carriers of germline mutations in the cancer susceptibility genes, BRCA1 and BRCA2, varies between individuals. It appears that hormonal factors (endogenous and exogenous) and cigarette smoking are both critical in modifying the risk. Estrogenic activity in pre-menopausal women is influenced by multiple factors, including lifestyle, hormonal medications and genetic variation in enzymes which metabolizes hormones. The gene for aryl hydrocarbon hydroxylase (CYP1A1) is involved in the metabolism of polyaromatic hydrocarbons and in the hydroxylation of estradiol. We have performed a registry based matched case-control study to evaluate whether or not allelic variations in CYP1A1 is associated with the risk of hereditary breast cancer.

We identified 209 cases with breast cancer and 209 matched controls. Controls were matched on year of birth, ethnic group and gene (BRCA1 versus BRCA2). Study subjects were limited to white women. Cases or controls who developed ovarian cancer prior to the age of diagnosis of breast cancer of the matched set where excluded. Controls who had a bilateral prophylactic mastectomy prior to the age of onset of the matched case were also excluded. CYP1A1 genotyping was performed on cases and controls. A single nucleotide polymorphism in the 3' region was identified by the Msp1 restriction enzyme.

The allelic variant was present in 14.4% of the cases and 21.5% of the controls. The odds ratio for breast cancer was estimated to be 0.62 associated with the CYP1a1 variant allele (P =.06), and was statistically significant for pre-menopausal cases (OR =0.58; P =.04). The distribution of the allele varied by ethnicity in the controls. 8.3% of French-Canadians carried the rare allele compared to 23.1% for Ashkenazi Jews and 23.3% for other caucasians. Because of this, we repeated the analysis excluding the French-Canadians. The odds for disease for the rare allele was 0.58 (P =.03) and was among pre-menopausal women 0.52 (P =.02).

248

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249

Cancer Risk Reduction by Prophylactic Surgery in BRCA1 and BRCA2 Mutation Carriers. *T. Rebbeck[1], A. Levin[1], M. Daly[2], J. Garber[3], C. Isaacs[4], C. Lerman[4], H. Lynch[5], S. Narod[6], S. Neuhausen[7], B. Weber[1].* 1) Biostatistics and Epidemiology, Univ of PA, Philadelphia, PA, USA; 2) Fox Chase Cancer Center, Philadelphia, PA, USA; 3) Dana Farber Cancer Institute, Boston, MA, USA; 4) Georgetown University, Washington, DC, USA; 5) Creighton Univ, Omaha, NE, USA; 6) Univ of Toronto, Tononto, ON, Canada; 7) University of Utah, Salt Lake City, UT, USA.

Genetic testing for inherited mutations in BRCA1 and BRCA2 provides potentially valuable information to women at high risk of breast or ovarian cancer. Unfortunately, carriers of inherited mutations in BRCA1 or BRCA2 have relatively few proven clinical management options available to reduce their cancer risk. We are undertaking a cohort study off BRCA1 and BRCA2 mutation carriers to evaluate the role of prophylactic surgery in reducing breast cancer risk and mortality.

Two groups of women are being studied. Surgical subjects are those women who have undergone a prophylactic mastectomy (PM) or prophylactic oophorectomy (PO). The occurrence of cancer in these women is compared with cancer occurrences in their sisters who have no history of PM or PO before the date of their sister's prophylactic surgery. These subjects are drawn from families with documented BRCA1 or BRCA2 mutations from seven participating sites (Creighton University, Dana-Farber Cancer Institute, Fox Chase Cancer Center, Georgetown University, University of Pennsylvania, University of Utah, Women's College Hospital). A total of 527 potentially eligible subjects with germline BRCA1 mutations have been identified in this preliminary data set— 154 (29%)with a history of mastectomy (78 contralateral and 76 bilateral), and 174 (33%) with a history of oophorectomy.

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1998 ASCO ABSTRACT FORM

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PROGNOSTIC FACTORS IN BRCA1- AND BRCA2-RELATED BREAST CANCER. D.K. GAFFNEY, B. LYNCH, J. HOLDEN, S. NEUHAUSEN, AND S. BUYS. The University of Utah, Salt Lake City, UT 84132

Patients with BRCA1-related breast cancer have an increased incidence of higher grade tumors and p53 mutations, while survival appears similar or improved when compared to patients with sporadic breast cancer. Fewer data exist regarding BRCA2-related breast cancer. To determine if prognostic factors could predict breast cancer relapses, we evaluated 18 BRCA1 cases and 12 BRCA2 cases for grade, stage, tumor size, age, S phase fraction, estrogen and progesterone receptors, topoisomerase II alpha, Mib1, p53 immunopositivity, Her2/neu, and Factor VIII. The median follow up interval was 74 months for the BRCA1 patients, and 71 months for the BRCA2 patients. Five of 18 patients in the BRCA1 group experienced a relapse with a mean time to recurrence of 51 months. Four of 12 BRCA2 patients relapsed with a mean time to recurrence of 38 months. Chi square analysis or t test was used to evaluate for statistical significance. In both the BRCA1 and BRCA2 group there was an increase in histologic grade and p53 immunopositivity compared with a sporadic control population; however, none of the examined prognostic factors were predictive of relapse. Although the sample sizes are small, these data suggest that despite BRCA1 and BRCA2 tumors having a higher histologic grade and greater frequency of p53 mutations, prognostic factors including grade, stage, tumor size, age, S phase, ER, PR, topoisomerase II alpha, Mib1, p53, Her2/neu, and factor VIII are not predictive of relapse free survival.

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1. FIRST AUTHOR / PRESENTER INFORMATION (please type):

Gaffney David K. M.D., Ph.D.
Last Name First Name Degree / Suffix

Institution University of Utah

50 N. Medical Dr., Salt Lake City, UT 84132
Address City, State, ZIP / Postal Code, Country

801-581-2396 801-585-3502 *
Telephone Number Fax Number E-mail Address

* david.k.gaffney@hsc.utah.edu

Signature: D Gaffney

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Estrogenic activity ... and genetic variation in ... lifestyle, hormonal medications and genetic variation in CYP1A1 is involved in the metabolism of mones. The gene for aryl hydrocarbon hydroxylase (CYP1A1) is involved in the metabolism of polyaromatic hydrocarbons and in the hydroxylation of estradiol. We have performed a registry based matched case-control study to evaluate whether or not allelic variations in CYP1A1 is associated with the risk of hereditary breast cancer.

We identified 209 cases with breast cancer and 209 matched controls. Controls were matched on year of birth, ethnic group and gene (BRCA1 versus BRCA2). Study subjects were limited to white women. Cases or controls who developed ovarian cancer prior to the age of diagnosis of breast cancer of the matched set where excluded. Controls who had a bilateral prophylactic mastectomy prior to the age of onset of the matched case were also excluded. CYP1A1 genotyping was performed on cases and controls. A single nucleotide polymorphism in the 3' region was identified by the Msp1 restriction enzyme.

The allelic variant was present in 14.4% of the cases and 21.5% of the controls. The odds ratio for breast cancer was estimated to be 0.62 associated with the CYP1a1 variant allele (P =.06), and was statistically significant for pre-menopausal cases (OR =0.58; P =.04). The distribution of the allele varied by ethnicity in the controls. 8.3% of French-Canadians carried the rare allele compared to 23.1% for Ashkenazi Jews and 23.3% for other caucasians. Because of this, we repeated the analysis excluding the French-Canadians. The odds for disease for the rare allele was 0.58 (P =.03) and was among pre-menopausal women 0.52 (P =.02).

Health, University ...

We analyzed pathology blocks from 1 ative breast cancer diagnosed at one November 1 1995. We tested the block by using PCR assays. Immunohistoc Clinicopathological data was extracted (13.5%) were found to carry *BRCA1* mut P53. *BRCA1*-mutation positive breast c (47.5 years vs. 54.8 years, p=0.02) and 1= 40, p=0.00004). The OR for P53 ov with non-carriers was 7.0 (95% confid deaths were due to breast cancer. 10 o *BRCA1* mutation carriers. The presence tage (eight year survival rate, 48.2% ve expressed P53 had a worse prognosis vival 65.5% versus 83.7%, p=0.046). group of women who, despite having status is available before diagnosis of b preventive and intensive or novel trea order to try to improve the survival rate

249

Cancer Risk Reduction by Prophylactic Surgery in BRCA1 and BRCA2 Mutation Carriers. *T. Rebbeck[1], A. Levin[1], M. Daly[2], J. Garber[3], C. Isaacs[4], C. Lerman[4], H. Lynch[5], S. Narod[6], S. Neuhausen[7], B. Weber[1].* 1) Biostatistics and Epidemiology, Univ of PA, Philadelphia, PA, USA; 2) Fox Chase Cancer Center, Philadelphia, PA, USA; 3) Dana Farber Cancer Institute, Boston, MA, USA; 4) Georgetown University, Washington, DC, USA; 5) Creighton Univ, Omaha, NE, USA; 6) Univ of Toronto, Tononto, ON, Canada; 7) University of Utah, Salt Lake City, UT, USA.

Genetic testing for inherited mutations in BRCA1 and BRCA2 provides potentially valuable information to women at high risk of breast or ovarian cancer. Unfortunately, carriers of inherited mutations in BRCA1 or BRCA2 have relatively few proven clinical management options available to reduce their cancer risk. We are undertaking a cohort study off BRCA1 and BRCA2 mutation carriers to evaluate the role of prophylactic surgery in reducing breast cancer risk and mortality.

Two groups of women are being studied. Surgical subjects are those women who have undergone a prophylactic mastectomy (PM) or prophylactic oophorectomy (PO). The occurrence of cancer in these women is compared with cancer occurrences in their sisters who have no history of PM or PO before the date of their sister's prophylactic surgery. These subjects are drawn from families with documented BRCA1 or BRCA2 mutations from seven participating sites (Creighton University, Dana-Farber Cancer Institute, Fox Chase Cancer Center, Georgetown University, University of Pennsylvania, University of Utah, Women's College Hospital). A total of 527 potentially eligible subjects with germline BRCA1 mutations have been identified in this preliminary data set— 154 (29%)with a history of mastectomy (78 contralateral and 76 bilateral), and 174 (33%) with a history of oophorectomy.

We have evaluated the role of prophylactic oophorectomy in decreasing breast cancer risk in BRCA1 mutation carriers. These preliminary analyses revealed a statistically significant reduction in breast cancer risk following bilateral prophylactic oophorectomy, with an adjusted hazard ratio estimate of 0.223 (95 %CI: 0.122-0.409). This suggests that prophylactic oophorectomy, presumably through reduction of ovarian hormone exposure, significantly reduces breast cancer risk in BRCA1 mutation carriers.

250

Germline ATM mutations in you

E. Solomon. Div. of Medical and Mo

Up to 1/100 individuals are heterc tasia.(AT). Studies of obligate AT carr ularly breast cancer in women, R.R =

We have designed a rapid protoc (ataxia telangiectasia mutated) for g ascertain whether AT heterozygosity tions. Nine overlapping products ar three fluorescent dyes. Three separa phase Fluorescent Chemical Cleava sequencer. This allows us to anal analysed by automated sequencin FCCM, confirming its sensitivity to c

Using this method we have scr for germline ATM mutations, 18 o (classified as an 'over-reactor', OR) have identified eight germline ATM putative missense mutation, 8293C domain, five rare variants and two s were identified in young breast can patients. Blood/tumour material fo being analysed for LOH using 5 m discussed. Preliminary data sugge in tumour tissue, showing that AT ants in this manner may help iden our understanding of the role of A